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Conjugated linoleic acid (CLA) is a potent inhibitor of experimentally-induced mammary carcinogenesis. Both chemically pure as well as naturally occurring forms of CLA exert this cancer preventive activity. While CLA can reduce levels of lipid peroxidation in the mammary gland, this apparent antioxidant effect may be secondary to a primary effect on CLA on suppressing the metabolism of linoleic acid. Suppression of linoleic acid metabolism may also account for the effects of CLA in decreasing mammary gland density and in inhibiting rates of cell proliferation in structural elements of the mammary gland that are targets for neoplastic transformation. Collectively, these data justify additional pre-clinical and clinical studies to evaluate the safety and efficacy of CLA for the prevention of human breast cancer.

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Introduction

Conjugated linoleic acid (CLA) is a constituent of "typical" Western type diets. This fatty acid is unique in that it has been shown to inhibit mammary carcinogenesis when fed at low concentrations (0.1-1.0% w/w) in the diet. The goal of this project was to determine the mechanism(s) by which CLA inhibits the development of experimental breast cancer. Our working hypothesis was that CLA affects the processes of clonal expansion and/or clonal selection via modulating genetic and/or epigenetic mechanisms obligatory for, or permissive to the carcinogenic process. This hypothesis was evaluated by determining the effect of CLA on the expression of molecular markers relevant to the process of mammary carcinogenesis. The following questions were addressed:***1) Does CLA inhibit the formation of oxidative damage to DNA?*** CLA has been reported to have potent antioxidant activity in test tube assay systems. It also has been shown that the c9,t11 isomer of CLA is selectively incorporated into membrane phospholipids of mammary epithelial cells. Together these observations form the rationale for evaluating the antioxidant activity of CLA in vivo. We determined whether CLA suppressed oxidative damage to mammary gland DNA by measuring the accumulation of 8-hydroxydeoxyguano-sine (8-OHG). Modification of this nucleoside has been implicated in site specific mutation of genes involved in carcinogenesis. ***2) Does CLA alter the process of clonal expansion that occurs in the mammary gland in response to carcinogenic insult?*** Our preliminary data indicated that CLA suppresses proliferation of some mammary gland components during the course of normal development, but it is unknown if CLA exerts a similar effect on transformed cells. We investigated whether CLA alters the process of clonal expansion of initiated mammary epithelial cells following carcinogen administration in vivo. ***3) Does CLA affect the process of clonal selection such that the pathogenetic pathway leading to mammary tumor formation is altered?*** Our hypothesis was that CLA inhibits tumor occurrence by modulating the "activity" of specific genes, whose misregulation is central to the carcinogenic process. Our goal was to identify these genes and whether CLA exerts a direct or an indirect effect on their expression.

6. Body

TECHNICAL OBJECTIVE 1. ***Does CLA inhibit the formation of oxidative damage to DNA?***
CLA has been reported to be a potent antioxidant in test tube assays but its biological activity as an antioxidant is unclear. The fact that the *c9,t11* isomer of CLA is selectively incorporated into membrane phospholipids of mammary epithelial cells indicates that CLA could exert a significant effect on oxidations that occur in membranes and/or are mediated via membrane constituents. We determined whether CLA alters oxidative damage to mammary gland DNA by measuring the accumulation of 8-hydroxydeoxyguanosine (8-OHG). Modification of this nucleoside has been implicated in site specific mutation of genes believed to affect the processes of clonal expansion and clonal selection.

Research Accomplishments Work on this technical objective has been summarized in three papers, two of which have been published and one of which is under review. These papers are provided in the appendix (Papers 4, 8, 9). In paper 4(Carcinogenesis 1996 May;17(5):1045-50), the hypothesis that CLA might act as an antioxidant was examined. Treatment with CLA resulted in lower levels of mammary tissue malondialdehyde (an end product of lipid peroxidation), but failed to change the levels of 8-hydroxydeoxyguanosine (a marker of oxidatively damaged DNA). Thus while CLA may have some antioxidant function in vivo in suppressing lipid peroxidation, its anticarcinogenic activity cannot be accounted for by protecting the target cell DNA against oxidative damage.

Based on this observation we then proceeded to further explore the effect of CLA on lipid peroxidation in vivo under conditions of increased oxidative stress. This work is reported in paper 9 which is under review. Two dietary lipids, fish oil (FO) and conjugated linoleic acid (CLA) , that have been shown to inhibit carcinogenesis in experimental animals, have also been reported to have opposite effects on lipid peroxidation. Lipid peroxidation has been hypothesized to be involved in the initiation, promotion, and progression stages of the carcinogenic process. The objective of this in vivo experiment was to determine the effect on lipid peroxidation when FO and CLA were fed alone or in combination. The study was designed to investigate this question under conditions of high oxidative stress. Sprague-Dawley rats were fed one of two basal diets, containing either menhaden oil (FO) or palm oil (PO) as the predominant lipid. Each diet was prepared both with and without CLA (1% w/w). Clofibrate was added to the diet (0.125%) to induce oxidative stress. Diets were fed for 4 wk. Liver MDA and 8-OHdG levels were observed to be elevated in rats fed the FO diet; CLA had no effect. The effect of FO and CLA on mammary gland levels of MDA was dramatic. FO increased mammary gland MDA by an order of magnitude ($p<0.01$), and CLA diminished significantly the extend of this increase ($p<0.05$). Thus the effects of FO and CLA on lipid peroxidation were opposing. The most prominent effects on lipid peroxidation were exerted in the mammary gland, a tissue which is rich in neutral lipid. This confirmed that the effect of CLA on lipid peroxidation was confined primarily to the mammary gland.

A partial explanation for the effects of CLA on lipid peroxidation was advanced in a recently published paper, paper 8 (Carcinogenesis 1999 Jun;20(6):1019-24). In this paper the ability of CLA to induce a marked decrease in linoleic acid metabolites is noted in mammary tissue, but not in liver. As reported above, we found that CLA feeding reduced malondialdehyde, an end product of lipid peroxidation, primarily in mammary gland, but not liver. Since only polyunsaturated fatty acids with three or more double bonds are degraded, via peroxidation, to malondialdehyde, our present observation of lower level of total linoleic acid metabolites is consistent with the depressed malondialdehyde levels seen in CLA-treated rats. Thus CLA could attenuate lipid peroxidation in cells by interfering with the formation of linoleic acid-derived polyunsaturated fatty acids which are the substrates for peroxidation. As such this would represent an indirect effect of CLA on fatty acid metabolism rather than a direct effect of CLA as an in vivo antioxidant.

Objective 2. ***Does CLA alter the process of clonal expansion that occurs in the mammary gland in response to carcinogenic insult?***

While preliminary data indicated that CLA suppresses proliferation of some mammary gland components during the course of normal development (Paper 1, Appendix), it was unknown whether CLA has any effect on the expansion of colonies of cells that are transformed, and how such an effect is exerted. We investigated whether CLA alters the process of clonal expansion of mammary epithelial cells *in vivo*.

Research Accomplishments Our initial efforts to study the effect of CLA on the process of clonal expansion focused on determining if CLA had an effect on the occurrence of carcinomas bearing the codon 12 mutation in Ha-ras. Before initiating these experiments it was necessary to answer key questions about Ha-ras-related pathogenetic characteristics of mammary carcinogenesis in our model systems. This work and the methods used are documented in Paper 12 in the appendix (Carcinogenesis 19: 223-227, 1998). The salient finds of that study are summarized below.

The induction of mammary carcinogenesis in the rat by 1-methyl-1-nitrosourea (MNU) is widely used in experimental breast cancer research. In the experiments reported, the Ha-ras codon 12 (ras12) mutation (GGA-->GAA) was used as a molecular marker to address issues of the clonality of carcinomas induced, pathogenetic independence among multiple carcinomas within the same animal and topographic distribution of mutant ras12 carcinomas in different mammary gland chains. In order to determine whether the frequently observed morphologically distinguishable lobules within carcinomas originate from the coalescence of independent lesions or whether cancerous cells within a carcinoma share a common origin, 44 randomly selected MNU-induced mammary carcinomas were genotyped for two to four lobules each for the ras12 mutation. A total of 43 carcinomas out of 44 (97.7%) had concordant ras12 genotypes among the multiple sites within each tumor, which is consistent with the latter possibility. Next, it was observed that as carcinoma multiplicity increased, the discordance rate of ras12 genotypes among multiple carcinomas within the same animal increased in a manner that was in excellent agreement with the expected discordance rate based on an assumption of no pathogenetic association among carcinomas. Furthermore, a significant difference was observed in the occurrence of mutant ras12 carcinomas between the cervical-thoracic and the abdominal-inguinal mammary glands in that three times as many carcinomas were mutant in the former as in the latter glands, whereas the occurrence of wild-type carcinomas was approximately the same in both regions. Taken together, the data are consistent with (i) carcinomas induced by MNU and detected by palpation are monoclonal in origin, (ii) independently-initiated cells emerge as distinct mammary carcinomas in the same animal, and (iii) the anatomical location of the gland may affect the prevalence of mammary carcinomas that harbor a mutant ras12.

Based on the above reported findings, we investigated whether CLA selectively inhibited clonal expansion of transformed mammary epithelial cells bearing a mutated Ha-ras gene. This work was reported in Carcinogenesis 18:755-759, 1997 (Paper 5, Appendix). Although continuous treatment with CLA reduced the total number of carcinomas by 70%, it did not alter the proportion of ras mutant versus wild-type carcinomas, suggesting that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of the ras mutation. This finding indicated that the approach originally proposed to measure the clonal expansion of mutant Ha-ras transformed foci would provide only limited insight. Therefore, we directed our effort to determining the effects of CLA on proliferation in the mammary gland.

The effects of CLA on cell proliferation in the mammary gland are summarized in Papers 6 and 8 in the Appendix. Our results showed that CLA treatment was able to cause a 20% reduction in the density of the ductal-lobular mammary tree as determined by digitized image analysis of the whole

mounts. This was accompanied by a suppression of bromodeoxyuridine labeling in the terminal end buds and lobuloalveolar buds. This observation is consistent with the hypothesis that exposure to CLA during the time of mammary gland maturation may modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation. In the study reported in Paper 8 we extended our initial observation. Feeding butter fat CLA to rats during the time of pubescent mammary gland development reduced mammary epithelial mass by 22%, decreased the size of the TEB population by 30%, suppressed the proliferation of TEB cells by 30% and inhibited mammary tumor yield by 53% ($P < 0.05$). Thus it is clear that CLA can inhibit the process of cell proliferation in the mammary gland, an effect that is correlated with its cancer preventive activity.

Objective 3. ***Does CLA affect the process of clonal selection such that the pathogenetic pathway leading to mammary tumor formation is altered?***

The hypothesis that forms the basis for this objective is that CLA inhibits tumor occurrence by modulating the "activity" of specific genes, whose misregulation is central to the carcinogenic process. A key point was to identify genes involved in the carcinogenic process, directly or indirectly, and that CLA modulates.

Research Accomplishments Efforts to identify candidate genes involved in carcinogenesis and that CLA might modify are reported in two papers in the Appendix. (Paper 10, Carcinogenesis 18:2085-2091, 1997 and paper 11, Molecular Carcinogenesis 20:204-215, 1997).

We had hypothesized that telomerase activity which appears to be a useful marker of breast cancer risk in the human, might be a candidate gene that CLA would modify. The work reported in paper 10 indicated that telomerase activity is not necessary for the immortalization phenotype in mouse mammary carcinogenesis. The acquisition of immortalization is an early and carefully documented event in mouse mammary tumorigenesis. Activation of telomerase activity is one hypothesis to explain the acquisition of immortalization. We examined the activity of telomerase in well-defined immortalized, non-tumor cell populations of mouse mammary tissue in vivo. The results indicated that normal virgin and mid-pregnant mammary gland had low to moderate levels of telomerase activity, respectively. In comparison with the levels detected in pregnant mammary gland, telomerase activity was elevated in mammary tumors in situ and in established mammary cell lines in vitro, both tumorigenic and nontumorigenic; however, hyperplastic alveolar preneoplastic mammary outgrowths and non-tumorigenic ductal outgrowths, both in vivo immortalized populations, had telomerase activity <12% of the levels detected in normal pregnant mammary gland. These results suggest that elevated telomerase activity is not necessary for the immortalization phenotype in in vivo mouse mammary cell populations and that elevated telomerase activity occurs as a late event in mammary tumorigenesis. Furthermore, the data suggest that low levels of telomerase activity are characteristic for mouse mammary epithelial cells and not sufficient for immortalization. These data suggest that other factors play more important roles in the induction and/or maintenance of the immortalization state in mammary cell populations. We further pursued this in parallel work in a chemically induced rat mammary carcinogenesis model (Appendix, Paper 13, Varon D, Jiang C, Hedican C, Dome JS, Umbricht CB, Carey LA, Thompson HJ, Sukumar S. *Cancer Res* 1997 Dec 15;57(24):5605-9). The 1-methyl-1-nitrosourea-induced rat mammary tumor model system is well studied, reproducible, and widely used. We have investigated whether these tumors possess higher telomerase activity than normal mammary tissue. Using the telomeric repeat amplification protocol assay, we found significantly higher telomerase activity in 36 mammary carcinomas than in 72 mammary glands of virgin rats. The level of telomerase activity in virgin rats was unaffected by strain, age, stage of the estrous cycle, or ovariectomy. However, mammary glands obtained from pregnant rats exhibited telomerase activity comparable to that found in the tumors, possibly reflecting the high epithelial

content of these tissues. Indeed, isolated epithelial cells from virgin and pregnant mammary glands and from carcinomas had similar telomerase activities. Thus, telomerase activity is constitutive in the rat mammary epithelium and is not a unique characteristic of malignant transformation in this tissue. These results underscore the importance of attributing biochemical properties to specific cell types in a tissue, a situation not paralleled in the interpretation of data from in vitro models. Based on these results we did not pursue studying the effects of CLA on telomerase activity.

In an effort to identify other candidate genes involved in MNU-induced mammary carcinogenesis an additional experiment was performed. The detailed results are reported in Paper 11 in the appendix. Briefly, experimentally induced models of breast carcinogenesis in the rat are widely used for studying the biology of breast cancer and for developing and evaluating cancer prevention and control strategies. However, very little is known about gene expression changes that are associated with experimentally induced mammary carcinogenesis. This paper reports the identification, by differential display of mRNA and molecular cloning, of seven cDNA fragments of gene transcripts overexpressed in mammary carcinomas induced by 1-methyl-1-nitrosourea. These genes included the rat homologues of human galectin-7 gene, the human/mouse melanoma inhibitory activity/bovine chondrocyte-derived retinoic acid sensitive protein gene, the mouse stearoyl-CoA desaturase-2 gene, and the mouse endo B cytokeratin/human cytokeratin-18 gene. Although each of these genes has been implicated in some aspect of carcinogenesis in other organs, this paper is the first report of their overexpression in chemically induced mammary carcinomas. Two previously uncharacterized gene transcripts were also identified. A comparison of the expression levels of several genes in mammary carcinomas with those in the normal mammary gland tissue of virgin rats, mid-stage pregnant rats, and of day 1 postpartum lactating dams indicated that the overexpression of several genes observed in mammary carcinomas could not be accounted for by either a difference in the mammary epithelial content between mammary carcinoma and normal mammary tissue or by mammary epithelium-specific proliferation associated with pregnancy. Several genes were also overexpressed in rat mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene but not in azoxymethane-induced rat colon adenocarcinomas. The genes identified in this study may therefore represent mammary carcinoma-specific molecular markers that may be helpful in investigations of mammary carcinogenesis and its prevention. Analysis of these transcripts provided no evidence to support a basis for CLA affecting their activity. Therefore, we did not pursue studying the effects of CLA on their expression.

We have engaged in a number of collaborations to facilitate the achievement of this technical objective. These collaborations have resulted in a series of publications that point to specific metabolic pathways that CLA is likely to be modifying and that result in its cancer preventive activity. The detail methods and results of these investigations are reported in appendix papers: 3, 4, 6, 7, and 8. Our findings are briefly summarized as follows. Initially a study was conducted (Paper 3) to verify that the anticancer activities of free fatty acid-CLA and triglyceride-CLA are essentially identical and they were. This was an important finding, because it ruled out a nonspecific free fatty acid effect. It was also observed that a continuous intake of CLA was required for maximal inhibition of tumorigenesis when CLA feeding was started after MNU administration, suggesting that some active metabolite(s) of CLA might be involved in suppressing the process of neoplastic promotion/progression. To follow up on this observation, the work reported in Paper 4 was conducted. The objective of the study was to investigate whether the anticarcinogenic activity of conjugated linoleic acid (CLA) is affected by the amount and composition of dietary fat consumed by the host. Because the anticancer agent of interest was a fatty acid, it was hoped that this approach would provide insight into its mechanism of action. For the fat level experiment, a custom formulated fat blend was used that simulates the fatty acid composition of the US diet. This fat blend was present at 10, 13.3, 16.7 or 20% by weight in the diet. For the fat type experiment, a 20% (w/w) fat diet

containing either corn oil (exclusively) or lard (predominantly) was used. Mammary cancer prevention by CLA was evaluated using the rat dimethylbenz[a]anthracene model. The results indicated that the magnitude of tumor inhibition by 1% CLA was not influenced by the level or type of fat in the diet. It should be noted that these fat diets varied markedly in their content of linoleate. Fatty acid analysis showed that CLA was incorporated predominantly in mammary tissue neutral lipids, while the increase in CLA in mammary tissue phospholipids was minimal. Furthermore, there was no evidence that CLA supplementation perturbed the distribution of linoleate or other fatty acids in the phospholipid fraction. Collectively these carcinogenesis and biochemical data raised the question of whether the cancer preventive activity of CLA was likely to be mediated by interference with the metabolic cascade involved in converting linoleic acid to eicosanoids, in contrast to the work reported in Paper 3. To resolve this issue additional work was done and is summarized in Papers 6, 7, 8.

The work reported in paper 6 showed the recovery of desaturation and elongation products of CLA in the mammary gland confirming our prior suggestion that the metabolism of CLA might be critical to risk modulation (Paper 3). The work reported in Paper 7 was based on the rationale that since both CLA and linoleic acid are likely to share the same enzyme system for chain desaturation and elongation, it is possible that increased CLA intake may interfere with the further metabolism of linoleic acid. Fatty acid analysis of total lipid showed that CLA and CLA metabolites continued to accumulate in mammary tissue in a dose-dependent manner over the range 0.5-2% CLA. There was no perturbation in tissue linoleic acid (as reported in paper 4), however, linoleic acid metabolites (including 18:3, 20:3 and 20:4) were consistently depressed by up to 1% CLA. Of particular interest was the significant drop in 20:4 (arachidonic acid), which is the substrate for the cyclooxygenase and lipoxygenase pathways of eicosanoid biosynthesis. Thus the CLA dose-response effect on arachidonic acid suppression corresponded closely with the CLA dose-response effect on cancer protection in the mammary gland. This information provided new insights regarding the biochemical action of CLA. These findings was confirmed in the work reported in Paper 8.

Key Research Accomplishments

- **CLA decreases lipid peroxidation in the mammary gland. This effect may be due to the modulation of linoleic acid metabolism by CLA rather than to an antioxidant effect per se.**

- **CLA results in a small reduction in the level of oxidative DNA damage, measured as 8-OHdG, in mammary tissue. However, the magnitude of this effect implies that a reduction of oxidative DNA damage by CLA is unlikely to account for its anticarcinogenic effect.**

- **CLA inhibits mammary carcinogenesis irrespective of the presence or absence of the Ha-ras mutation.**

- **CLA results in a reduction in the density of the epithelial component of the mammary gland, an effect that is due at least in part to a decrease in the rate of cell proliferation. Breast density is a marker of breast cancer risk.**

- **CLA decreases the number of mammary terminal end buds which are a cell type highly susceptible to neoplastic transformation by chemical carcinogens in both animals and humans. CLA also reduced the rate of cell proliferation within mammary terminal end buds. These effects of CLA on mammary gland development are associated with a decreased carcinogenic response attributable to short term feeding of CLA during the period of rapid mammary gland development in the maturing rat.**

- **CLA resulted in a significant drop in mammary tissue arachidonic acid, which is the substrate for the cyclooxygenase and lipoxygenase pathways of eicosanoid biosynthesis. The CLA dose-response effect on arachidonic acid suppression corresponded closely with the CLA dose-response effect on cancer protection in the mammary gland.**

8. Reportable Outcomes

Ip, C., Scimeca, J. And Thompson, H.J. Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer prevention. Nutrition and Cancer 24:241-247, 1995.

Ip, C., Briggs, S.P., Haegele, A.D., Thompson, H.J., Storkson, J., and Scimeca, J.A. The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. Carcinogenesis: 17 1045-1050, 1996.

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Thompson, H.J., Zhu, Z., Banni, S., Darcy, K., Loftus, T., and Ip, C. Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: implication for a reduction in mammary cancer risk. Cancer Res. 57: 5067-5072, 1997

Lu, J., Pei, H. Kaeck, M. and Thompson, H.J. Gene expression changes associated with chemically induced rat mammary carcinogenesis. Mol. Carcinogenesis 20: 204-215, 1997.

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9. Conclusions

Conjugated linoleic acid (CLA) is a potent inhibitor of experimentally-induced mammary carcinogenesis. Both chemically pure as well as naturally occurring forms of CLA exert this cancer preventive activity. While CLA can reduce levels of lipid peroxidation in the mammary gland, this apparent antioxidant effect may be secondary to a primary effect on CLA on suppressing the metabolism of linoleic acid. Suppression of linoleic acid metabolism may also account for the effects of CLA in decreasing mammary gland density and in inhibiting rates of cell proliferation in structural elements of the mammary gland that are targets for neoplastic transformation. Collectively, these data justify additional pre-clinical and clinical work to evaluate the safety and efficacy of CLA for the prevention of human breast cancer.

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Project specific

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APPENDIX

[CANCER RESEARCH 54, 1212–1215, March 1, 1994]

Paper 1

Conjugated Linoleic Acid Suppresses Mammary Carcinogenesis and Proliferative Activity of the Mammary Gland in the Rat[1]

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ABSTRACT

Conjugated linoleic acid (CLA) is a collective term which refers to a mixture of positional and geometric isomers of linoleic acid. It is naturally occurring in meat and dairy products. We have previously reported (Ip, C., Chin, S. F., Scimeca, J. A., and Pariza, M. W. Cancer Res., *51:* 6118–6124, 1991) that 1% CLA in the diet suppressed mammary carcinogenesis in rats given a high dose (10 mg) of 7,12-dimethylbenz(a)anthracene. In the present study, dietary CLA between 0.05 and 0.5% was found to produce a dose-dependent inhibition in mammary tumor yield when fed chronically to rats treated with a lower dose (5 mg) of 7,12-dimethylbenz-(a)anthracene. Short-term CLA feeding for 5 weeks, from weaning to the time of carcinogen administration at 50 days of age, also offered significant protection against subsequent tumor occurrence. This period corresponds to maturation of the mammary gland to the adult stage in the rat. The inhibitory response to short-term CLA exposure was observed with the use of 2 different carcinogens: 7,12-dimethylbenz(a)anthracene and methylnitrosourea. The fact that CLA was protective in the methylnitrosourea model suggests that it may have a direct modulating effect on susceptibility of the target organ to neoplastic transformation. The proliferative activity of the mammary epithelium was assessed by the incorporation of bromodeoxyuridine. Immunohistochemical staining results showed that CLA reduced the labeling index of the lobuloalveolar compartment, but not that of the ductal compartment of the mammary tree. Since the lobuloalveolar structures are derived from the terminal end buds which are the sites of carcinogenic transformation, the above finding is consistent with the bioassay data of tumor inhibition. Thus, changes in gland development and morphogenesis may be a locus of action of CLA in modulating mammary carcinogenesis. CLA is a unique anticarcinogen because it is present in foods from animal sources. Furthermore, its efficacy in cancer protection is manifest at dietary concentrations which are close to the levels consumed by humans.

INTRODUCTION

A voluminous amount of data is available in the literature linking increased consumption of fat and stimulation of mammary tumorigenesis in the animal model. This subject has been thoroughly discussed in several review articles (1, 2). The mechanisms that might account for the enhancing effect of fat have yet to be resolved. A high fat diet is also a calorie-dense diet. Thus, the relationship between dietary fat and mammary cancer could potentially be complicated by changes in energy intake. Any digestible fat at levels beyond that required for cellular homeostasis and structural integrity may serve as a source of excess calories, and it is this increased metabolizable energy that is conducive to the proliferation of cancer. There is, however, a specific effect of fat which relates to individual fatty acids. For example, linoleic acid has been consistently associated with enhancement of mammary cancer development in rodents (2). In contrast to linoleic acid, we have previously reported that isomers of linoleic acid, denoted as conjugated linoleic acid, are able to prevent mammary tumorigenesis induced by a carcinogen (3).

Linoleic acid is an essential polyunsaturated fatty acid of 18-carbon chain length with 2 double bonds in the 9 and 12 positions (both are in the *cis* configuration). CLA[3] is a collective term which refers to a mixture of positional and geometric isomers of linoleic acid. The 2 double bonds in CLA are in positions 9 and 11, or 10 and 12, along the carbon chain, thus giving rise to the designation of a conjugated diene. Each of the double bonds can be in the *cis* or *trans* configuration. CLA, a normal isomerization metabolite of linoleic acid by rumen bacteria (4), is a naturally occurring substance in food. It was initially isolated and identified by Ha *et al.* (5) as an anticarcinogenic agent from grilled ground beef. These investigators subsequently showed that cheese is also a rich source of CLA (6). Rumen bacteria are unlikely to be the sole producer of CLA found in unprocessed food, since raw meat from nonruminants (*e.g.*, pork, chicken, and turkey) is known to contain measurable but lower amounts of this fatty acid (7). Cooking has been shown to increase the concentration of CLA in meat (6). However, the mechanism of linoleic acid conversion to CLA during cooking and food processing remains to be clarified.

In an earlier publication, we reported that 1% by weight of CLA in the diet maximally suppressed mammary carcinogenesis in rats given a 10-mg dose of DMBA (3). This was the first study demonstrating that chronic CLA feeding, from 2 weeks before DMBA administration until the end of the experiment, was effective in cancer prevention. The work reported in this paper was designed to: (*a*) evaluate the dose dependency of dietary levels of CLA in the range between 0.05 and 0.5% for mammary cancer inhibition in rats given a low dose of DMBA (5 mg); and (*b*) determine whether short-term CLA feeding from weaning (21 days of age) to the time of carcinogen administration (50 days of age) was able to offer protection against subsequent tumor development. This particular period corresponds to maturation of the rat mammary gland to the adult stage morphology (8). The effects of CLA on DMBA binding to mammary cell DNA as well as proliferative indices of the mammary epithelial component were also investigated to gain insight into whether alterations in these parameters might contribute to changes in cancer susceptibility.

MATERIALS AND METHODS

Mammary Tumor Induction by Carcinogen. Pathogen-free weanling female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and housed in a room with a 12-h light/12-h dark cycle. Mammary tumors were induced by the administration of a carcinogen at 50 days of age. In Experiment 1, all rats were given a p.o. intubation of 5 mg of DMBA (Sigma, St. Louis, MO) dissolved in 1 ml of corn oil. There were 50 rats/group. This sample size ensured adequate statistical power due to the reduced number of tumors produced per rat by the low dose of carcinogen. In Experiment 2, 2 different carcinogens were used. Rats were given either 10 mg of DMBA p.o. or 6 mg of MNU (Ash Stevens, Inc., Detroit, MI) i.p. There were 25 rats/group. All animals were palpated weekly to determine the appearance and location of tumors. Experiments 1 and 2 were terminated at 36 and 24 weeks, respectively, after carcinogen administration. At necropsy, the mammary glands were exposed for the detection of nonpalpable tumors. Only confirmed adenocarcinomas were reported in the results. Tumor incidences at

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: CLA, conjugated linoleic acid; DMBA, 7,12-dimethylbenz(a)anthracene; MNU, methylnitrosourea.

the final time point were compared by χ^2 analysis, and the total tumor yield between groups was compared by frequency distribution analysis as described previously (9). The statistical analyses of intergroup differences in tumor incidence and number were corrected for multiple comparisons.

Dietary Treatment and CLA Supplementation. Rats were acclimatized immediately to the powdered AIN-76A diet (10) upon arrival. One modification was made in this standard formulation. A mixture of dextrose and corn starch (1:1 ratio) was substituted for sucrose as the carbohydrate source. In Experiment 1, different levels of CLA (0.05, 0.1, 0.25, and 0.5% by weight) were added to the basal diet. The CLA-containing diets were given to the animals starting at 2 weeks before DMBA administration and continuing for 36 weeks until the end of the experiment. In Experiment 2, a diet containing 1% CLA was given to the rats from weaning until 1 week past carcinogen administration (*i.e.*, for a total of 5 weeks). The animals were then returned to the basal diet without CLA for the duration of the experiment. For the DMBA binding and the mammary gland bromodeoxyuridine labeling studies, which will be described below, the CLA feeding protocol was similar to that of Experiment 2, *i.e.*, starting from weaning and continuing for a period of 5 weeks, after which the animals were sacrificed.

The method of CLA synthesis from 99+% pure linoleic acid has been detailed in our earlier publication (3). The CLA used in the present studies was custom ordered from Nu-Chek Prep, Inc. (Elysian, MN). Gas chromatography analysis of the CLA preparation showed the following composition: *c*9,*t*11- and *t*9,*c*11-CLA, 43.3%; *t*10,*c*12-CLA, 45.3%; *c*9,*c*11-CLA, 1.9%; *c*10,*c*12-CLA, 1.4%; *t*9,*t*11- and *t*10,*t*12-CLA, 2.6%; linoleic acid (unchanged parent compound), 4.4%; and remainder (unidentified), 1.4%. The chemical composition of CLA from Nu-Chek was very similar to that prepared at the Kraft General Foods Technology Center and which was used in our previous studies (3).

DMBA Binding to Mammary Cell DNA. Rats were fed either the basal or 1% CLA diet from weaning. At 50 days of age, they were given 10 mg of ^{3}H-labeled DMBA (1 mCi/rat; Amersham) i.g. and were sacrificed 1, 2, 4, or 7 days later. There were 4 rats/time point. Mammary glands were excised and immediately dropped in liquid nitrogen. Frozen mammary tissue was pulverized and mammary epithelial aggregates were dissociated from adipocytes and stromal materials by collagenase digestion. The methodologies involved in mammary cell DNA isolation, purification, quantitation, and the determination of bound DMBA by liquid scintillation counting have been described in detail in a previous publication by Ip and Daniel (11).

Bromodeoxyuridine Labeling of the Mammary Gland. Rats were fed either the basal or 1% CLA diet from weaning (n = 15/group). For the last 3 days of the 5-week CLA feeding period, rats were given 5 i.p. injections of bromodeoxyuridine at 12-h intervals. A total of 58.6 μmol were administered per rat, and the proportion of labeled cells was detected by immunohistochemical staining using the procedure described by Eldridge *et al.* (12). Rats were euthanized 12 h after the final bromodeoxyuridine injection. The thoracic mammary glands were rapidly excised and fixed in methacarn. Cells that incorporated bromodeoxyuridine were identified by brown to black granules over the nuclei. One thousand consecutive ductal nuclei were counted, and the number that stained positive was noted. The same procedure was followed for the lobuloalveolar nuclei. The level of positive labeling in each mammary compartment was expressed as a percentage.

RESULTS

Dose Response of Dietary CLA in Inhibition of Mammary Tumorigenesis. In this experiment, rats were fed a diet containing 0.05, 0.1, 0.25, or 0.5% of CLA starting 2 weeks before DMBA and continuing for 9 months. With a 5-mg dose of DMBA, tumors take a longer time to develop and usually begin to level off by 8–9 months after carcinogen administration. The time course of cumulative tumor yield in the control and CLA-treated groups is shown in Fig. 1. The complete tumor data and their statistical analysis are summarized in Table 1. Two types of analysis were done with the data. (*a*) The entire data set for the different levels of CLA was analyzed as a whole to look for a dose-dependent effect. (*b*) Each dietary level of CLA was compared to the control in order to find out the particular level of CLA at which inhibition of tumorigenesis first became statistically significant. A dose-dependent inhibition of mammary carcinogenesis by CLA was observed in the range between 0.05 and 0.5%. The progressive decreases in tumor incidence ($P < 0.05$; Table 1, Footnote *b*) and total tumor yield ($P < 0.02$; Table 1, Footnote *c*) as a function of increasing dietary CLA levels were analyzed by logistic regression and polynomial regression, respectively. Intergroup comparisons showed that as little as 0.1% CLA was sufficient to cause a significant reduction in the total number of tumors ($P < 0.05$; Table 1, Footnote *d*). Thus, this study convincingly demonstrated that the administration of CLA via the dietary route is an effective way of achieving cancer protection.

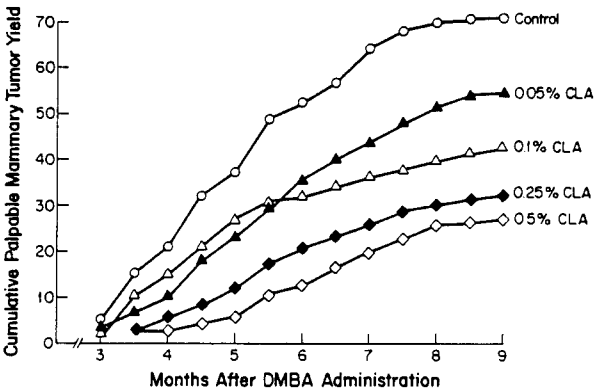

Fig. 1. Cumulative appearance of palpable mammary tumors as a function of time after DMBA administration in rats fed different levels of CLA.

Table 1 *Mammary cancer prevention by supplementation of different levels of CLA in the diet*[a]

% dietary CLA supplementation	Tumor incidence[b] (%)	Total no. of mammary tumors[c]	Final body wt (g)
None	28/50 (56)	74	367 ± 10
0.05	29/50 (58)	58	371 ± 9
0.1	21/50 (42)	47[d]	370 ± 10
0.25	17/50 (34)[d]	37[d]	374 ± 11
0.5	18/50 (36)[d]	31[d]	369 ± 9

[a] Rats were given 5 mg of DMBA i.g. CLA was present in the diet starting at 2 weeks before DMBA and continuing for 36 weeks until the end of the experiment.
[b] Change in tumor incidence response to 0.05 to 0.5% CLA analyzed by logistic regression, $P < 0.05$.
[c] Change in mammary tumor yield response to 0.05 to 0.5% CLA analyzed by polynomial regression ($P < 0.02$).
[d] $P < 0.05$ compared to the corresponding control value.

In our previous publication with the DMBA model (3), we reported that dietary CLA at 1.5% failed to produce any change in growth rate, food intake, or organ weight (liver, spleen, kidney, and uterus). Also discussed in the paper was an independent pathology examination study in which rats were fed 1.5% CLA for 36 weeks, although they were not treated with DMBA. There was no evidence of histomorphological abnormality observed in any one of 15 different tissues. The current experiment used lower levels of CLA, and again we did not detect any change in growth of the animals (data not shown). This is mentioned to affirm that reproducible results were obtained with different batches of CLA. The growth curves from all 5 groups would have been superimposable if they were plotted out graphically. Their body weights at necropsy are included in Table 1 to corroborate that CLA is a safe and effective anticarcinogen which can be consumed chronically without any apparent adverse effect on the host.

Mammary Cancer Prevention by Short Term Feeding of CLA. The period from weaning to about 50 days of age in the rat (the time of carcinogen administration) corresponds to maturation of the mammary gland to the adult stage with the number of terminal end buds decreasing gradually and differentiating to alveolar buds and lobules (8). It should be noted that carcinogenic initiation of the rodent mammary model occurs primarily in the epithelium of the terminal end

buds. We wanted to see whether limiting CLA feeding to this particular time frame would offer any protection against subsequent tumorigenesis. Two different carcinogens were used in this experiment for mammary tumor induction: DMBA, which requires metabolic activation, and MNU, which is a direct alkylating agent. Rats were fed 1% CLA from weaning to 1 week past carcinogen treatment, they were then returned to the basal diet without CLA until sacrifice. Our results in Table 2 showed that CLA exposure during this narrow window of mammary gland morphogenesis was able to significantly reduce total mammary tumor yield by 39 and 34% in the DMBA and MNU models, respectively. Based on the results of the first experiment, the addition of 1% CLA would be expected to produce a greater suppressive effect. However, it should be taken into consideration that the administration of a high dose of carcinogen for mammary tumor induction in this experiment (see Table 2 for doses of DMBA and MNU), coupled with the shorter duration of CLA feeding, probably accounted for the attenuated inhibitory response. But more importantly, the fact that CLA is protective in the MNU model suggests that it may have a direct modulating effect on susceptibility of the target organ to neoplastic transformation.

Effect of CLA Feeding on Bromodeoxyuridine Labeling of the Mammary Gland. To follow up on the above observations, we carried out an experiment to evaluate the effect of CLA on proliferative activity of the mammary gland. CLA feeding was started from weaning and continued until about 55 days of age. Essentially the protocol was identical to the previous one with the exception that no carcinogen was used in this experiment. Multiple injections of bromodeoxyuridine, as described in "Materials and Methods," enable a higher proportion of cells to be labeled by this biochemical marker and thereby increases the sensitivity of the assay. The results in Table 3 show that CLA reduced the labeling index of the lobuloalveolar compartment ($P < 0.05$), but not that of the ductal compartment of the mammary tree. The significance of this finding will be discussed below.

Effect of CLA on DMBA Binding to Mammary Cell DNA. It is possible that the lower proliferative activity of the mammary gland following CLA feeding may not fully explain the increased resistance of the target organ to carcinogenesis. In order to determine whether the inhibitory response in the DMBA model to CLA feeding prior to carcinogen administration might be related to changes in DMBA activation, total DMBA binding to mammary epithelial cell DNA was measured at different times after a single dose of radioactive DMBA. The results in Table 4 indicate conclusively that total DMBA binding was not affected by CLA. Although these data do not rule out the possibility that differences may exist in the formation of specific DMBA-DNA adducts, our past experience has suggested that total DMBA binding is a fairly reliable indicator of changes in DMBA activation and therefore of adduct formation.

Table 2 *Mammary cancer prevention by short term feeding of CLA*[a]

Carcinogen[b]	% dietary CLA supplementation	Tumor incidence (%)	Total no. of mammary tumors
DMBA	None	20/25 (80)	62
DMBA	1	13/25 (52)[c]	38[c]
MNU	None	22/25 (88)	76
MNU	1	15/25 (60)[c]	50[c]

[a] CLA was added to the diet starting from weaning until 1 week past carcinogen for a total period of 5 weeks.
[b] Rats were given 10 mg of DMBA i.g. or 6 mg of MNU i.p. at 50 days of age.
[c] $P < 0.05$ compared to the corresponding control value.

Table 3 *Effect of CLA feeding on proliferative activity of ductal and lobuloalveolar mammary epithelial cells*[a]

Dietary CLA supplementation	No.	% mammary epithelial component[b]	
		Ductal	Lobuloalveolar
None	15	25.7 ± 3.1	32.4 ± 2.6
1%	15	21.9 ± 2.5	24.8 ± 2.9[c]

[a] CLA was added to the diet starting from weaning and continuing for the next 5 weeks before excision of the mammary gland.
[b] Results are expressed as percent of cells (mean ± SE) labeled by bromodeoxyuridine.
[c] $P < 0.05$.

Table 4 *Effect of CLA feeding on total DMBA binding to mammary cell DNA*[a]

Dietary CLA supplementation (%)	Time course of total DMBA binding[b] (pmol/mg DNA)			
	Day 1	Day 2	Day 4	Day 7
None	33 ± 5.2	40 ± 6.9	31 ± 5.0	22 ± 3.8
1%	31 ± 6.1	38 ± 4.7	36 ± 3.6	25 ± 3.4

[a] Rats were fed either the basal or 1% CLA diet from weaning. At 50 days of age, they were given 10 mg of ^{3}H-labeled DMBA and were sacrificed at 1, 2, 4, or 7 days later.
[b] Measured by total tritium binding to DNA after ^{3}H-labeled DMBA administration. Results are expressed as mean ± SE (n = 4/group).

DISCUSSION

Of the large number of naturally occurring substances that have been demonstrated to have anticarcinogenic activity in experimental animal models, all but a handful of them are of plant origin (13). CLA is unique because it is present preferentially in food from animal sources. CLA is closely related to linoleic acid, but differs from linoleic acid in the position and configuration of the double bonds. Unlike the stimulatory effect of linoleic acid on mammary carcinogenesis (14), CLA inhibits tumor development. As shown in Table 1, as little as 0.1% of CLA in the diet is sufficient to produce a significant reduction in mammary tumor yield in rats given a low dose of DMBA. The low dose carcinogen protocol was intended to increase the sensitivity of the animal bioassay so that the efficacy of CLA could be titrated more precisely. A 350-g rat fed a 0.1% CLA diet will consume about 0.015 g CLA/day. In a direct extrapolation to a 70-kg person, this is equivalent to a daily CLA intake of 3 g, an amount slightly higher than the estimated consumption of approximately 1 g/person/day in the United States (6). In the much quoted nurses' study by Willet *et al.* (15), dietary fat was found not to be a risk factor for breast cancer. Although the range of fat intake in this cohort was not as broad as that observed in international ecology studies, which generally report a positive correlation between breast cancer mortality and fat intake (16), it is tempting to speculate that the presence of CLA in the Western diet may play some role in moderating the impact of high fat consumption on breast cancer risk. Preliminary experiments from our laboratory indeed suggest that CLA could negate the stimulatory effect of fat on mammary carcinogenesis in the rat DMBA model.[4] Thus, the cancer protective efficacy of CLA needs to be further characterized in order to fully delineate its contribution to health maintenance and disease control.

Scanty information is available in the literature on the anticarcinogenic activity of CLA *in vivo*. To date, only 3 papers have been published regarding CLA supplementation in animal tumor models. The 2 papers from Ha *et al.* (5, 17) reported successful tumor inhibition in skin and forestomach of mice treated with a carcinogen. In both cases, CLA was delivered acutely in single doses prior to either DMBA (for initiation of skin papillomas) or benzo(a)pyrene (for induction of forestomach tumors) administration. Our previous study with the rat DMBA mammary tumor model was the first to show that dietary CLA is effective in cancer protection (3). Experiments will be

[4] C. Ip, and J. A. Scimeca, unpublished observations.

under way to contrast the action of CLA in the initiation and post-initiation phases of mammary carcinogenesis.

In the present study, we failed to detect changes in total DMBA binding to mammary epithelial cell DNA following CLA feeding. Although definitive conclusion about DMBA activation still needs to be confirmed by the determination of specific DMBA-DNA adducts, we feel that this might not be a promising avenue to pursue future mechanistic investigation. Total DMBA binding was measured at 4 different time points over a span of 7 days (Table 4). No difference was noticeable between the control and CLA-fed animals during this period, suggesting that the kinetics of DMBA metabolism were not affected. Urinary DMBA levels were similarly unchanged (data not shown). By day 7 after DMBA, there was virtually no more radioactive DMBA being excreted in the urine. The lack of an effect on DMBA removal would be consistent with our previous finding that CLA has no effect on glutathione-S-transferase or UDP-glucuronyl transferase activities (3). Based on these observations, it seems that neither Phase I nor Phase II detoxification enzymes may be the targets for the action of CLA in events associated with modulation of DMBA-induced mammary carcinogenesis.

As shown in Table 3, CLA reduced the proliferative activity of the lobuloalveolar structures of the mammary gland, but not of the ducts. This difference, however, was small and needs to be confirmed. Since the determination was made at a single time point, it is unclear whether this outcome is due to a change in the time course of mammary gland maturation or a quantitative shift in gland composition. Multiple time point analysis on both kinetic and morphological parameters would be necessary in order to address this question. Similar studies should also be carried out with and without carcinogen administration. In the absence of any information correlating the effect of CLA on proliferation and differentiation during maturation of the mammary gland, it is not possible to make a definitive conclusion regarding the impact of CLA on susceptibility of the target organ to carcinogenesis. Nonetheless, suffice it to note that the lobuloalveolar structures are derived from the terminal end buds which are the sites of carcinogenic transformation. The observation that the proliferative activity of the lobuloalveolar units is reduced by CLA feeding is congruent with the bioassay results of tumor inhibition. What is so tantalizing about this finding is that exposure to CLA during mammary gland morphogenesis in the adolescent period (30 to 60 days of age in a rat) may be able to provide lasting protection against subsequent cancer risk. In humans, early onset of puberty increases, but early pregnancy decreases, the risk of breast cancer (18). Both factors highlight the importance of developmental changes of the mammary gland during the period of adolescence in determining the risk of breast cancer. Further investigation will be focused on examining the role of CLA in influencing mammary gland morphogenesis and proliferation.

The subject of CLA and cancer prevention is in its infancy. In the previous paper as well as the present one, we have identified several areas that are critical in expanding the horizon of CLA research. CLA is by far the most powerful naturally occurring fatty acid known to modulate tumorigenesis. The fact that a fatty acid can produce such a striking effect at near nutritional levels of intake is fascinating. However, foods that are high in CLA are also high in fat content. Consequently, it is difficult to evaluate from epidemiology data the impact of CLA alone in the context of a high fat intake in humans. Much emphasis has been placed on total fat consumption and breast cancer risk, perhaps justifiably so. On the other hand, the equivocal and often negative results from case-control and cohort studies have hinted at the complexity of this issue (16). The challenge of future research will be to define the potential benefit of CLA in our diet, to characterize its anticancer activity, to elucidate its mechanism of action at the subcellular level, and to design new strategies for enriching foods with CLA if this approach is deemed appropriate.

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Conjugated Linoleic Acid

A Powerful Anticarcinogen from Animal Fat Sources

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Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid, which is found preferentially in dairy products and meat. Preliminary studies indicate that CLA is a powerful anticarcinogen in the rat mammary tumor model with an effective range of 0.1–1% in the diet. This protective effect of CLA is noted even when exposure is limited to the time of weaning to carcinogen administration. The timing of this treatment corresponds to maturation of the mammary gland to the adult stage, suggesting that CLA may have a direct effect in reducing the cancer risk of the target organ. Of the vast number of naturally occurring substances that have been demonstrated to have anticarcinogenic activity in experimental models, all but a handful of them are of plant origin. Conjugated linoleic acid is unique because it is present in food from animal sources, and its anticancer efficacy is expressed at concentrations close to human consumption levels. *Cancer* 1994; 74:1050–4.

Key words: conjugated linoleic acid, mammary cancer prevention, mammary gland development, CLA incorporation in mammary cells.

Interest in fat and experimental mammary cancer research has been fueled by the availability of a variety of animal models. There is a voluminous amount of data linking increased consumption of fat, particularly linoleic acid, and stimulation of mammary tumorigenesis.[1,2] Linoleic acid is an essential polyunsaturated fatty acid of 18-carbon chain length with two double bonds in the 9 and 12 positions (both are in the *cis* configuration). It is present predominantly in vegetable oils such as corn oil and safflower oil. Previous work by Carroll and Hopkins[3] hinted at a specific requirement of linoleate for mammary carcinogenesis in rodents. In the early 1980s, Ip and associates carried out a large scale experiment aimed at titrating the sensitivity of the dimethylbenz(a)anthracene (DMBA)-induced mammary tumor model in rats to increasing dietary levels of linoleate. They found that the yield of mammary tumors progressed linearly in proportion to an increasing intake of linoleate from 0.5 to 4% by weight.[4] There was no further stimulation above 4%, suggesting that a maximal response was reached at this point. This level of linoleate clearly is beyond the nutritional requirement for growth and maintenance of the animal. A more recent study by Fischer et al.[5] with the DMBA-induced mammary tumor model in SENCAR mice also showed a positive dose–response relationship when animals were fed a diet containing linoleate in the range of 0.8–8%.

The relationship between dietary fat and cancer is complicated by many factors, especially with respect to total caloric intake. Suffice it to note that a high fat diet is also a calorie-dense diet. A few years ago, Donato and Hegsted[6] reemphasized the concept that a calorie from fat is not physiologically equivalent to a calorie from protein or carbohydrate because of the increase in energy use from fat. Consequently, animals may retain more energy from a high fat diet than from a low fat diet. Fat and calories are so tightly linked in the human diet that it is difficult to study these two effects separately. This problem can only be resolved in an experimental model in which the consumption of fat and calories can be individually controlled. By using specially formulated isonutrient diets and adopting a graded feeding restriction protocol, Ip was able to design a series of animal experiments that were aimed at delineating the impact of fat and calories as independent risk factors in mammary carcinogenesis. His study, therefore, provided some measure of a quantitative assessment of risk changes as effected by a decrease in fat or calorie intake.[7] He found that calorie restriction, even in the presence of a high fat intake, is more striking than a decrease in dietary fat in suppressing the development

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of mammary cancer, suggesting that total energy consumption is a more important determinant than dietary fat in altering mammary cancer risk.

In view of the above discussion, it appears that there may be a dual effect of fat on mammary carcinogenesis. The first is general, in that any digestible fat at levels beyond that required for metabolic homeostasis and structural integrity may serve as a source of excess calories. The second is specific and relates to individual fatty acids. Under the latter premise, linoleic acid clearly is associated with enhancement of mammary cancer development. This presentation, however, is focused on characterizing the cancer protective action of conjugated linoleic acid, a newly recognized anticarcinogenic fatty acid that is found predominantly in animal food sources.

Food Sources and Cancer Preventive Activity of Conjugated Linoleic Acid

Conjugated dienoic derivative of linoleic acid, abbreviated as CLA, is a collective term that refers to a mixture of positional and geometric isomers of linoleic acid (*c*9, *c*12-octadecadienoic acid). The two double bonds in CLA are in positions 9 and 11, or 10 and 12, along the carbon chain, thus giving rise to the designation of a conjugated diene. Each of the double bonds can be in the *cis* or *trans* configuration. Theoretically, eight possible geometric isomers of 9, 11- and 10,12-octadecadienoic acid (*c*9, *c*11-; *c*9, *t*11-; *t*9, *c*11-; *t*9, *t*11-; *c*10, *c*12-; *c*10, *t*12-; *t*10, *c*12-; *t*10, *t*12-) may form from the isomerization of linoleic acid. CLA is a naturally occurring substance in food. It initially was isolated and identified by Pariza and coworkers as an anticarcinogenic agent from grilled ground beef[8] and then shown also to be present in a variety of dairy products.[9] Virtually all of the CLA in milk and cheeses is esterified in triglycerides. However, the CLA content of cheeses varies considerably, ranging from approximately 3 to 9 mg/g of fat.[10,11] Part of the reason for this variability can be attributed to the processing conditions. Pariza and coworkers developed methods for quantifying different isomers of CLA in foods. They found that of the mixture of isomers, the *c*9, *t*11-octadecadienoic acid accounted for more than 80% of total CLA in a variety of cheeses.[10]

CLA is a normal isomerization product of linoleic acid metabolism by rumen bacteria. The pathway has been studied extensively in the anaerobe, *Butyrivibrio fibrisolvens*.[12] In beef, 75% or more of the CLA is in the form of the *c*9, *t*11-isomer, which is believed to be the biologically active form. This selective accumulation is consistent with the finding that rumen bacteria preferentially isomerize linoleic acid to the *c*9, *t*11 configuration.[13] Rumen bacteria may not be the only source for the biosynthesis of CLA, because it has recently been shown that pork and chicken also contain CLA. Nonetheless, meat from ruminants generally contains more CLA than meat from nonruminants. The former group is estimated to contain 3–6 mg CLA/g of fat, whereas the latter has less than 1 mg CLA/g of fat.[10] Cooking increases the concentration of CLA in meat. For example, the CLA content in ground beef is increased by almost 5-fold after grilling.[9] The mechanism of linoleic acid conversion to CLA during cooking and food processing is unknown; however, many factors may be involved, including the oxidative environment, temperature, and protein quality of the product.[9]

Scanty information is available in the literature on the anticarcinogenic activity of CLA. Pariza and coworkers initially examined chemically prepared CLA for antiinitiation activity in the two-stage mouse epidermal carcinogenesis model.[8] CLA was topically applied at 7 days (at a dose of 20 mg/mouse), 3 days (20 mg) and 5 minutes (10 mg) before DMBA treatment. Control mice instead were painted with linoleic acid before DMBA administration. All mice were given 12-O-tetradecanoylphorbol-13-acetate for tumor promotion. It was found that CLA reduced the number of papillomas by half compared with that in the linoleic acid-treated control subjects. In a second study performed by the same investigators, the synthetic CLA mixture decreased benzo(a)pyrene-induced forestomach tumors in mice by about 50%.[14] A dose of 0.1 ml of CLA was administered by gavage in this experiment at 4 and 2 days before treatment with benzo(a)pyrene during the first week, and this sequence was repeated for 4 consecutive weeks. We recently have reported a rat mammary cancer prevention experiment in which CLA was administered in the diet.[15] Our design contrasted with the acute dosing of CLA as described in the above two papers and was intended to simulate human intake of CLA. This and other experiments will be described below.

Mammary Cancer Prevention by CLA in Rats Treated with a High or Low Dose of DMBA

We have used primarily the dimethylbenz(a)anthracene (DMBA)-induced mammary tumor model in female Sprague–Dawley rats for our studies. The CLA preparation used in these experiments was synthesized by refluxing linoleic acid (99 + % pure) with sodium hydroxide in a nitrogen atmosphere.[15] Gas chromatographic analysis of the CLA methyl ester derivatives showed a mixture of eight isomers. However, three particular isomers, i.e., *c*9, *t*11-, *t*9, *c*11-, and *t*10, *c*12-octadecadienoates, accounted for about 85% of the total. Different amounts of CLA, in the form of free fatty

acids, were added directly to the basal AIN-76A diet, containing 5% corn oil. The basal diet is practically free of CLA.

In the initial study, three levels of CLA feeding (0.5, 1, and 1.5% by weight) were started 2 weeks before DMBA administration and continued until the end of the experiment (6 months after DMBA administration). All rats (n = 30 per group) received 10 mg of DMBA (high dose) by gavage at approximately 50 days of age. We fully realized that this design would not allow us to differentiate the effect of CLA on the initiation versus the promotion stage of carcinogenesis. On the other hand, we had no a priori knowledge of whether CLA would be effective in cancer prevention under a chronic feeding schedule or in this particular model. On this basis, we decided to expose the animals to CLA before, during, and after DMBA treatment to maximize our ability to detect a protective effect of CLA. Results of this study have been reported in our previous publication.[15]

Our data indicated that the total number of mammary tumors in the 0.5, 1, and 1.5% CLA groups was reduced by 32, 56, and 60%, respectively. The final tumor incidence and cumulative tumor weight were diminished similarly in rats fed the CLA-containing diets. In general, there appeared to be a dose-dependent protection at levels of 1% CLA and below, but no further beneficial effect was evident at levels above 1%. Chronic feeding of CLA produced no adverse consequences in the animals. Growth rate, food intake, and organ weights were normal in all groups. An independent experiment also was performed in which 1.5% CLA was fed to rats for 36 weeks (no DMBA treatment). At the end of the experiment, a pathologist evaluated 15 different tissues and found no evidence of histomorphologic abnormality. Thus, CLA appears to be a safe and effective anticarcinogen that can be consumed at relatively high levels.

Because of certain constraints in the design described above, such as a high dose of carcinogen (10 mg of DMBA) and a modest sample size (n = 30), it was necessary to use a generous quantity of CLA to produce a significant reduction in cancer risk. In an attempt to expand the CLA efficacy curve below 0.5%, we performed another experiment similar to the previous one with the exception of two modifications: (1) animals were given 5 mg of DMBA (low dose), and (2) the sample size per group was increased to 50. The larger sample size would ensure adequate statistical power because of the reduced number of tumors produced per rat by the low dose of carcinogen. Rats were fed the CLA-containing diets at 0.05, 0.1, 0.25, and 0.5% starting 2 weeks before DMBA administration and continuing for 9 months. Tumors took a longer time to develop with the low dose of DMBA.

There was clearly a dose-dependent effect of CLA on mammary cancer inhibition, with a range of 0.05–0.5% ($P < 0.05$ by regression analysis). Total mammary tumor yield was reduced by 22, 36, 50, and 58% in the 0.05, 0.1, 0.25, and 0.5% CLA diets, respectively. Intergroup comparison showed that as little as 0.1% CLA was sufficient to cause a significant reduction in the number of tumors. Thus, not only have we been able to confirm that CLA is a powerful anticarcinogen, we also have shown conclusively that the administration of CLA via the dietary route is an effective way of achieving cancer protection.

Incorporation of CLA in Mammary Cells

The mammary gland from a virgin rat is basically a fatty tissue, consisting primarily of adipocytes, fibroblasts, stromal material, but few epithelial cells. Fat in adipocytes is stored in the form of triglycerides. Previous work has shown that all isomers of CLA appear in triglycerides, whereas only the *c*9, *t*11-isomer is found in membrane phospholipids.[14,15] The phospholipid fraction of the whole mammary gland constitutes only about 1% of the total amount of extractable fat. Unless isolated and purified mammary epithelial cells are obtained first, it is technically difficult to analyze for the small amount of specific incorporation of the *c*9, *t*11-isomer in the phospholipid fraction of the mammary gland because of potential contamination by the triglycerides that are present in much larger proportion in the whole mammary tissue.

An interesting question is whether the *c*9, *t*11-CLA isomer is incorporated in membrane phospholipids of the mammary epithelial cells. In our preliminary study, mammary epithelial cells were dissociated from the whole gland by digestion with collagenase and dispase. The digested tissue was pelleted (upon centrifugation, the fat cells floated to the top of the tube), washed, and filtered through a series of different-sized nitex filters to trap the epithelial organoids that then were placed in a plastic culture flask and incubated briefly to facilitate the attachment and subsequent removal of stromal contaminants. We recently have worked out the conditions for pooling and harvesting a sufficient number of purified mammary epithelial cells from the whole gland so that CLA incorporation in phospholipids can be accurately measured. We extracted the phospholipids by silicic acid column chromatography and then sent the samples to Dr. Michael Pariza's laboratory at the University of Wisconsin for the high pressure liquid chromatography and gas chromatography work. Dr. Sou-Fei Chin performed the actual analysis.

Preliminary results indicated that in rats fed the basal AIN-76A diet, no CLA was detectable at all in the phospholipids of mammary epithelial cells. In rats fed 1% CLA, the incorporation of the *c*9, *t*11-isomer was approximately 1.5 to 2.0 μg/mg phospholipid. No other isomer was found besides the *c*9, *t*11-isomer. Increases in *c*9, *t*11-CLA incorporation were not achieved at the expense of linoleic acid, suggesting that there is a certain degree of specificity involved in the process. Because the anticancer efficacy of CLA covers a range of 0.1–1% in the diet, it is important to determine whether the CLA content in phospholipids of mammary epithelial cells increases in proportion to dietary intake. In other words, is phospholipid CLA in the target organ a reliable marker for tissue resistance to carcinogenesis? We plan to continue this line of investigation in the near future.

What is the physiologic significance of CLA incorporation in the mammary gland in relation to the inhibition of tumorigenesis? The fact that only the *c*9, *t*11-isomer is incorporated into membrane phospholipids of mammary epithelial cells suggests a possible locus of action associated with the signal transduction pathway. This mechanism could involve changes in responsiveness to peptide hormone stimulatory and/or inhibitory factors, which are known to play an important role in regulating the proliferation, morphogenesis, differentiation, and transformation of mammary epithelial cells. In addition, CLA is a potent antioxidant.[14] Because adipocytes are an integral component of the mammary gland, the accumulation of CLA (all isomers) in triglycerides of fat cells may bestow some insulation against oxidant stress in the microenvironment of the epithelial cell habitat. These are the areas that will be the primary focus of our future research plan.

Mammary Cancer Prevention by Short-Term Feeding of CLA

In the previous CLA cancer prevention experiments, CLA was administered at 2 weeks before DMBA and then continued for 6–9 months. We wanted to see whether short-term CLA feeding from weaning (21 days of age) to the time of carcinogen administration was able to offer any protective effect against subsequent tumorigenesis. This particular period, i.e., from weaning to about 50 days of age, corresponds to the maturation of the rat mammary gland to the adult stage with the number of terminal end buds decreasing gradually and differentiating to alveolar buds and lobules. Carcinogenic initiation of the rat mammary model occurs primarily in the epithelium of the terminal end buds.[16] Two different carcinogens were used in our experiments for mammary tumor induction: DMBA, which requires metabolic activation, and methylnitrosourea, which is a direct alkylating agent. Our results showed that 1% CLA significantly suppressed total mammary tumor yield by 39 and 34% in the DMBA and methylnitrosourea models, respectively, when it was supplemented in the diet for a duration of approximately 5 weeks (from weaning to 1 week after carcinogen administration). Because CLA is inhibitory in the methylnitrosourea model suggests that it may have a direct modulating effect on the susceptibility of the target organ to carcinogenesis.

To follow-up on this line of investigation, we decided to do some preliminary work regarding the effect of CLA on proliferative activity of the mammary gland. Rats were fed a diet containing 1% CLA from weaning until 55 days of age. No carcinogen was administered in this experiment. One week before sacrifice, animals were implanted with a bromodeoxyuridine pellet. The continuous release of bromodeoxyuridine by this method enables a higher proportion of cells to be labeled by this marker and, therefore, increases the sensitivity of the assay. The incorporation of bromodeoxyuridine into proliferating cells was quantitated by an immunostaining technique. We found that feeding of CLA reduced markedly (about 25% lower) the proliferative activity of the lobuloalveolar compartment of the mammary tree.

Conclusion and Implication of Research

Of the vast number of naturally occurring substances that have been demonstrated to have anticarcinogenic activity in experimental models, all but a handful of them are of plant origin.[17] CLA is unique because it is present in food from animal sources. Thus, in terms of novelty, CLA provides an example that fats from meat and dairy products contain some component that has an attribute in cancer protection.

Perhaps it would be informative to put in perspective the potency of CLA relative to other fatty acids that have been known to modulate tumorigenesis. Conjugated linoleic acid is related closely to linoleic acid, but differs from linoleic acid in the position and configuration of the double bonds. But unlike the stimulatory effect of linoleic acid on carcinogenesis, CLA inhibits tumor development. Fish oil is a class of lipid that has been reported by many investigators to inhibit both chemically induced and transplantable tumors.[18] Eicosapentaenoic acid and docosahexaenoic acid are probably the major n-3 fatty acids in fish oil responsible for tumor suppression. However, the amount of fish oil needed to elicit this response usually exceeds 10% in the diet. As shown in this presentation, a level of CLA as low as 0.1% was sufficient to produce a significant

reduction in mammary tumor yield in rats challenged with a low dose of DMBA. Thus, the effect of CLA in cancer prevention is specific; moreover, CLA is more powerful than any other fatty acid in modulating tumor development.

A 300-g rat fed a 0.1% CLA diet consumes about 0.015 g of CLA per day. In a direct extrapolation to a 70-kg person, this is equivalent to a daily CLA intake of 3.5 g, a figure slightly higher than the estimated consumption of approximately 1 g/person/day in the United States.[9] If the cancer protective efficacy of CLA can be characterized further and its mechanism of action delineated in the near future, there is a good possibility that a CLA-enriched food product may serve as a prototype of a new generation of designer food. This approach may be particularly appealing to people who are unwilling to change their eating habits but still desire alternative food choices for cancer prevention.

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Effect of Timing and Duration of Dietary Conjugated Linoleic Acid on Mammary Cancer Prevention

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Abstract

Conjugated linoleic acid (CLA) is a minor fatty acid found predominantly in the form of triglycerides in beef and dairy products. Previous work by Ip and co-workers showed that free fatty acid-CLA at ≤1% in the diet is protective against mammary carcinogenesis in rats. The present study verified that the anticancer activities of free fatty acid-CLA and triglyceride-CLA are essentially identical. This is an important finding, because it rules out a nonspecific free fatty acid effect. In terms of practical implication, we can continue the in vivo *research with the less-expensive free fatty acid-CLA without compromising the physiological relevance of the data. A primary objective of this report was to investigate how the timing and duration of CLA feeding might affect the development of mammary carcinogenesis in the methylnitrosourea (MNU) model. We found that exposure to 1% CLA during the early postweaning and pubertal period only (from 21 to 42 days of age) was sufficient to reduce subsequent tumorigenesis induced by a single dose of MNU given at 56 days of age. This period incidentally corresponds to a time of active morphological development of the mammary gland to the mature state. In contrast to the above observation, a continuous intake of CLA was required for maximal inhibition of tumorigenesis when CLA feeding was started after MNU administration, suggesting that some active metabolite(s) of CLA might be involved in suppressing the process of neoplastic promotion/progression.*

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Introduction

Conjugated linoleic acid (CLA) is a collective term that refers to a mixture of positional and geometric isomers of linoleic acid (1). The two double bonds in CLA are in Positions 9 and 11 or 10 and 12 along the carbon chain, thus giving rise to the designation of a conjugated diene. Each of the double bonds can be in the *cis (c)* or *trans (t)* configuration. CLA is normally found as a minor constituent in the lipid fraction of many different kinds of food (2). Meat from ruminants generally contains more CLA than meat from nonruminants. Cheese and other dairy products are also good sources of CLA, whereas seafoods and vegetable oils are not. Although the biochemistry of CLA has been documented for decades in the literature, little is known about its nutritional activity or requirement (3). More than 30 years ago, Bartlett and Chapman (4) first reported that CLA was an intermediate in the microbial biohydrogenation of linoleic acid

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in butter fat. Kepler and associates (5) subsequently discovered that a rumen bacterium, *Butyrivibrio fibrisolvens,* was able to convert linoleic acid to stearic acid via CLA. Microorganisms are not necessarily the major producer of CLA. Additional factors may facilitate the formation of CLA in cooked and processed foods. For example, grilling ground beef has been shown to increase the CLA content in beef fat by about fourfold (1). The new era of CLA and cancer prevention research began after the identification by Pariza's laboratory (6–8) of an antimutagenic and anticarcinogenic substance isolated in grilled ground beef.

In contrast to linoleic acid, which has been observed consistently to enhance mammary tumorigenesis in rodents over a wide concentration range (9–11), CLA expresses an inhibitory effect at ≤1% in the diet (12,13). The studies demonstrating a cancer-promoting effect of dietary linoleic acid were often conducted using vegetable oils (e.g., corn oil or safflower oil), which are rich in linoleate esterified to glycerol. CLA is likewise present naturally as a component of triglyceride in food. However, CLA was given as a free fatty acid in the previous animal mammary cancer prevention experiments (12,13). Triglyceride-CLA is not routinely used in vivo because of the prohibitive cost. However, the question has remained open as to whether the free fatty acid-CLA effect could be artifactual. One of the objectives of this study therefore was to compare the cancer-preventive efficacy of triglyceride-CLA with that of free fatty acid-CLA to evaluate the relevance of the latter form in biologic research.

Scanty information is available concerning the effect of interrupted vs. continuous CLA feeding on the benefit of cancer protection. As a first step in addressing this question, a major focus of the present report was to examine how the timing and duration of CLA feeding might affect the risk of mammary cancer development. All the experiments described below were carried out using the methylnitrosourea (MNU)-induced mammary tumor model in rats. MNU is a direct alkylating agent and does not require metabolic activation. Because the design involved CLA feeding immediately before or after carcinogen treatment, the MNU model is ideal for this purpose, inasmuch as it obviates any potential confounding influence of CLA on carcinogen metabolism.

Materials and Methods

Source of CLA

The method of CLA synthesis from >99% pure linoleic acid was detailed previously (12). The free fatty acid-CLA was custom ordered from Nu-Chek (Elysian, MN). Gas chromatographic analysis (12) showed the following composition: *c*9,*t*11- and *t*9,*c*11-CLA, 42.6%; *t*10,*c*12-CLA, 44.8%; *c*9,*c*11-CLA, 2.1%; *c*10,*c*12-CLA, 1.4%; *t*9,*t*11- and *t*10,*t*12-CLA, 2.8%; linoleic acid (unchanged parent compound), 1.8%; unidentified remainder, 4.5%.

A portion of this batch was set aside by Nu-Chek to prepare the triglyceride-CLA. The procedure involved reacting CLA methyl ester with triacetin in the presence of a sodium methoxide catalyst. Triglyceride-CLA was then separated from the mono- and diglycerides on silicic acid pads by repeated washings with petroleum ether-hexane. Because Nu-Chek has proprietary right to this procedure, no further information on the methodology can be released. High-pressure liquid chromatographic analysis of the triglyceride-CLA at the Kraft Foods Technology Center confirmed a purity of >99%. In addition, fatty acid analysis of the triglyceride-CLA indicated an isomer distribution nearly identical to that shown above, suggesting insignificant isomerization during the synthesis of the triglyceride form. CLA was the only fatty acid present in the synthetic triglyceride.

Design of Mammary Cancer Chemoprevention Experiments

Pathogen-free female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and housed in a room with a 12:12-hour light-dark cycle. Mam-

mary tumors were induced by a single injection of MNU (Ash Stevens, Detroit, MI; 50 mg/kg body wt ip). The MNU solution was prepared following the method of Thompson and Adlakha (14). Animals were palpated weekly to determine the time of appearance and location of tumors. At necropsy, the mammary glands were exposed for the detection of nonpalpable tumors. Only histologically confirmed adenocarcinomas were reported in the results. Tumor incidences at the final time point were compared by χ^2 analysis, and the total tumor yield between groups was compared by frequency distribution analysis, as described previously (15). Three mammary carcinogenesis experiments were carried out in accordance with the goals outlined in the **Introduction.**

The first experiment was designed to compare the efficacy of free fatty acid-CLA with that of triglyceride-CLA. Both forms of CLA were added to a modified basal AIN-76A diet (12) at a concentration of 1% by weight. The CLA-containing diets were fed from weaning until 56 days of age, when the animals were injected with MNU. CLA was removed at this point, and the rats were maintained on the basal AIN-76A diet until sacrifice (22 wks post-MNU). Control rats were fed the basal AIN-76A diet without CLA from weaning but were otherwise treated similarly.

The second experiment was designed to confirm that exposure to CLA during the period of active mammary gland development was critical for subsequent cancer protection. Animals were fed the 1% free fatty acid-CLA diet from weaning and were injected with MNU at 42 or 56 days of age. As in the above experiment, CLA was removed from the diet after MNU administration. The appropriate control rats were injected with MNU at either of the two time points but were fed the basal AIN-76A diet without CLA for the duration of the experiment.

The third experiment was designed to investigate the effect of different durations of CLA feeding after MNU treatment. Animals were injected with MNU at 56 days of age and were then given the 1% free fatty acid-CLA diet for one month, two months, or continuously until sacrifice (5 mos post-MNU).

Results

Table 1 summarizes the results of mammary cancer prevention by free fatty acid-CLA or triglyceride-CLA. In this experiment, the CLA diets were fed from weaning until 56 days of age (the time of MNU treatment), i.e., a duration of five weeks. Both forms of CLA were effective in tumor suppression. Furthermore the magnitude of the inhibitory effect was almost identical with the two reagents, suggesting that the free fatty acid form of CLA was absorbed as efficiently as the triglyceride form. This is an important observation, because it rules out a nonspecific free fatty acid effect. Additionally, it also means that the less-expensive free fatty acid-CLA can be used in animal feeding studies without compromising the interpretation of our findings.

The rat mammary gland undergoes marked morphological changes during the five-week

Table 1. Comparison of the Efficacy of Free Fatty Acid-CLA and Triglyceride-CLA in Mammary Cancer Prevention[a,b]

Dietary Treatment	Tumor Incidence	Total No. of Tumors
Control	24/30 (80.0%)	69
1% Triglyceride-CLA	16/30 (53.3%)*	37*
1% Free fatty acid-CLA	15/30 (50.0%)*	35*

a: Both forms of conjugated linoleic acid (CLA) were supplemented in the diet starting at weaning (21 days of age) and continuing until the time of methylnitrosourea administration at 56 days of age.
b: Statistical significance is as follows: *, $p < 0.05$ compared with corresponding control.

Table 2. Mammary Cancer Prevention by CLA Feeding From Weaning to the Time of MNU Administration[a–c]

Dietary Treatment	Age of MNU Administration, Days	Tumor Incidence	Total No. of Tumors
Control	42	25/30 (83.3%)	78
1% CLA	42	17/30 (56.7%)*	41*
Control	56	26/30 (86.7%)	81
1% CLA	56	16/30 (53.3%)*	47*

a: Animals were weaned at 21 days of age.
b: Free fatty acid-CLA was used in this experiment.
c: Statistical significance is as follows: *, $p < 0.05$ compared with corresponding control.

period after weaning (16). The above experiment suggests that exposure to CLA within this critical window of gland development is able to confer a lasting protective effect against mammary carcinogenesis in the absence of sustained treatment. Thompson and colleagues (17) recently showed that treatment with MNU as early as 28 days of age produced essentially the same carcinogenic response, as measured by tumor incidence and number. To verify a direct effect of CLA on the mammary gland, a second experiment was undertaken in which rats were injected with MNU at 42 or 56 days of age and the CLA diet was fed from weaning (21 days of age) to the time of MNU treatment. On the basis of the information from the first experiment, a 1% free fatty acid-CLA diet was used. Thus the length of CLA feeding was limited to three or five weeks, respectively, before MNU in the two supplemented groups. The results (Table 2) clearly indicate that exposure to CLA during the early postweaning and adolescent life span of the rat is sufficient in reducing the susceptibility of the mammary gland to subsequent carcinogen-induced neoplastic transformation. Rats injected with MNU at 42 days of age were maintained on the CLA diet for only three weeks, and yet the tumor-inhibitory activity of CLA with this protocol was very comparable to that observed with five weeks of CLA feeding (MNU injection at 56 days of age).

The last phase of the research was aimed at determining the effect of CLA feeding on the postinitiation phase of mammary carcinogenesis. CLA (1% as free fatty acid) was given immediately after MNU administration (dosed at 56 days of age) and was maintained for one month, two months, or continuously until termination of the experiment. The time course of tumor development in the different groups is shown in Figure 1. It is evident that short-term exposure to CLA for one or two months post-MNU was relatively ineffective in cancer protection. Significant inhibition ($p < 0.05$) was observed only in the group that received an uninterrupted supply of CLA in the diet.

Discussion

Although CLA has been shown to have a marked cancer-inhibitory activity (12,13), there is essentially no information about how it works or the conditions in which it is effective. In this regard, there are a number of noteworthy observations in the present study. First, the data in Table 1 indicate that the cancer-inhibitory activities of free fatty acid-CLA and triglyceride-CLA are very similar. This resolves the question of whether the previous reports of CLA's protective activity might be a nonspecific effect resulting from the feeding of CLA as a free fatty acid. The above observation also has practical value, in that it demonstrates the validity of continuing the future *in vivo* work with the more affordable free fatty acid-CLA without compromising interpretation of the data within the boundary of human relevance. Second, our experiments indicated that the timing of CLA feeding is clearly important in modulating mammary cancer risk. As demonstrated by the results summarized in Tables 1 and 2, exposure to CLA during the early postweaning and adolescent period is sufficient in

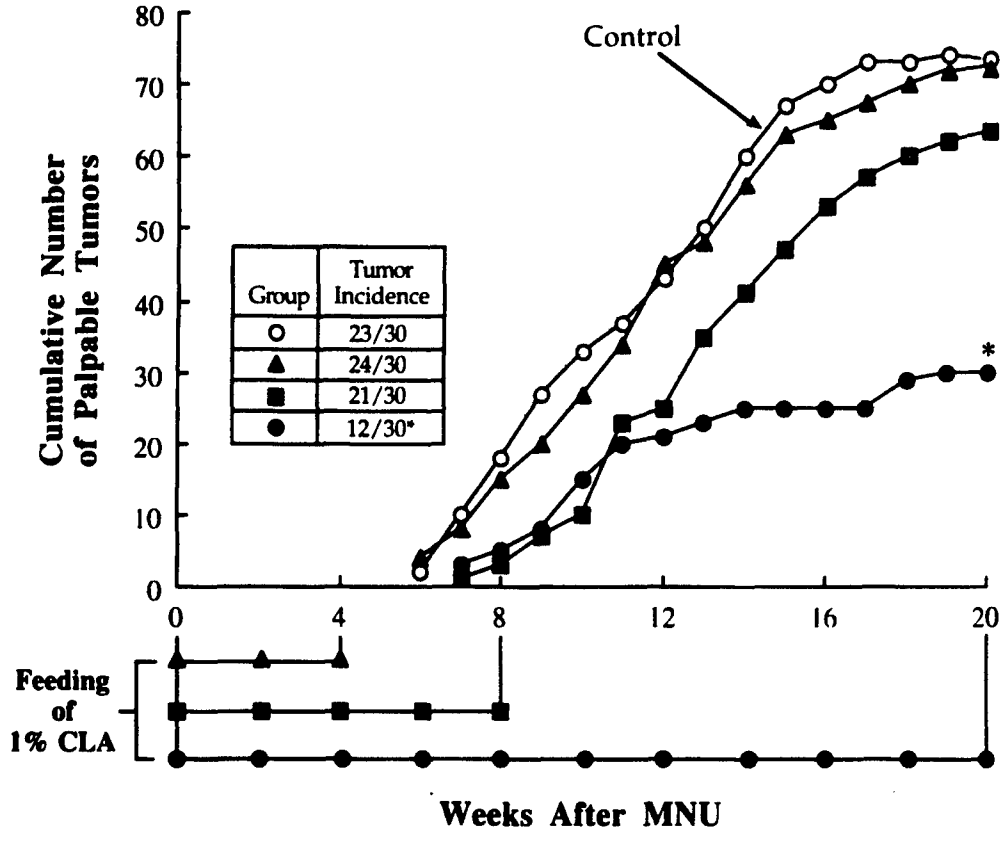

Figure 1. Effect of interrupted vs. continuous conjugated linoleic acid (CLA) feeding after methylnitrosourea (MNU) administration on mammary carcinogenesis. Duration of CLA feeding in 3 supplemented groups is indicated along *x*-axis time line by *filled symbols*, which match time course of mammary tumor development on main body of diagram. *Open circles*, control group without CLA supplementation. *, Statistically significant difference ($p < 0.05$) from control.

conferring some measurable degree of protection against subsequent chemically induced tumorigenesis in the mammary gland. This window of opportunity encompasses only a few weeks in the life span of the rat and yet appears to furnish certain epigenetic changes in rendering the target tissue less susceptible to neoplastic transformation. Third, it is evident from the experiment illustrated in Figure 1 that once the mammary cells have been initiated by a carcinogen, a continuous intake of CLA is required to achieve maximal inhibition of tumorigenesis.

According to the work of Russo and Russo (16), the rat mammary gland undergoes extensive morphological remodeling during the postnatal, prepubertal, and pubertal periods. At birth and in the first week of postnatal life, the mammary gland is made up of a single primary duct, which branches into several secondary ducts. These ducts end in dilated club-shaped structures called terminal end buds. During the second and third weeks, additional sprouting of the ducts occurs, leading to a sharp increase in the number of terminal end buds. After they reach a peak at weaning (21 days of age), the terminal end buds begin to reduce markedly in size and number because of their differentiation to alveolar buds and lobules. By about 40 days of age, these latter structures are far more prevalent than the terminal end buds and their population density is approaching a morphological state seen in the mature gland. We reported previously that CLA suppressed the level of bromodeoxyuridine labeling in the lobuloalveolar fraction (13). The determination was made at about 55–60 days of age after five weeks of CLA feeding, i.e., at a time when there were few terminal end buds left. Chemically induced

mammary carcinomas in the rat are believed to originate from terminal end bud cells and not from lobuloalveolar cells (16). Although it is reasonable to assume that the proliferative activity of the lobuloalveolar cells may be indicative of that of the progenitor terminal end bud cells, further work is clearly necessary to delineate the role of CLA in modulating the kinetics of mammary gland development. In view of our present observation that the feeding of CLA from weaning to 42 days of age (a duration of only 3 wks) is capable of protecting the mammary gland against tumorigenesis (Table 2), it is critical to find out whether this protective effect is due to 1) a general decrease in proliferative activity during gland morphogenesis, 2) a change in the time course of gland maturation, leading to a quantitative shift in gland composition, or 3) a specific response of target epithelial cells and/or the stromal component that surrounds the epithelial structures.

For a rational elucidation of how CLA might interfere with carcinogenesis after the mammary cells have been initiated, it might be profitable to consider the metabolic disposition of CLA *in vivo*. Being a fatty acid, CLA could potentially be 1) metabolized for energy, 2) incorporated as a component of membrane phospholipids and neutral lipids, or 3) converted to some other biologically active substances. The first route is unlikely to be of interest in the context of cancer prevention, whereas little is known about the significance of the latter two alternatives. We have unpublished data indicating that, in rats fed a 1% CLA diet, CLA is found in the phospholipid and neutral lipid fractions of the mammary epithelial cells. Our preliminary results also show that the incorporation of CLA as a percentage of total fatty acids is much higher in neutral lipids (~3%) than in phospholipids (~0.4%), suggesting that some selectivity might be involved in the compartmentalization of CLA into different classes of lipids. The presence of CLA in membrane phospholipids could conceivably be linked to the signal transduction pathway. This mechanism may modify the responsiveness to peptide stimulatory and/or inhibitory factors, which are known to play an important role in regulating the proliferation, morphogenesis, differentiation, and transformation of mammary epithelial cells. With respect to the neutral lipid CLA, it is possible that this pool may serve as the precursor to some as yet unidentified oxidized metabolites in the target tissue. Oxidation products of linoleic acid, including hydroperoxy-, hydroxy-, and oxooctadecadienoic acid, have been shown to express potent biologic activity in a number of different systems (18–20). It is possible that similar oxidation metabolites are produced from CLA. As illustrated by the data in Figure 1, the rate of tumor appearance rose after a short delay upon withdrawal of CLA feeding. This observation added weight to the idea of some active metabolites that are dependent on the availability of CLA. The intracellular effects of CLA might be multifocal. Our objective at this point is to suggest certain potentially fruitful areas of research that could contribute to the understanding of the mechanism of action of CLA in cancer prevention.

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The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet

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The objective of the present study was to investigate whether the anticarcinogenic activity of conjugated linoleic acid (CLA) is affected by the amount and composition of dietary fat consumed by the host. Because the anticancer agent of interest is a fatty acid, this approach may provide some insight into its mechanism of action, depending on the outcome of these fat feeding experiments. For the fat level experiment, a custom formulated fat blend was used that simulates the fatty acid composition of the US diet. This fat blend was present at 10, 13.3, 16.7 or 20% by weight in the diet. For the fat type experiment, a 20% (w/w) fat diet containing either corn oil (exclusively) or lard (predominantly) was used. Mammary cancer prevention by CLA was evaluated using the rat dimethylbenz[*a*]anthracene model. The results indicated that the magnitude of tumor inhibition by 1% CLA was not influenced by the level or type of fat in the diet. It should be noted that these fat diets varied markedly in their content of linoleate. Fatty acid analysis showed that CLA was incorporated predominantly in mammary tissue neutral lipids, while the increase in CLA in mammary tissue phospholipids was minimal. Furthermore, there was no evidence that CLA supplementation perturbed the distribution of linoleate or other fatty acids in the phospholipid fraction. Collectively these carcinogenesis and biochemical data suggest that the cancer preventive activity of CLA is unlikely to be mediated by interference with the metabolic cascade involved in converting linoleic acid to eicosanoids. The hypothesis that CLA might act as an antioxidant was also examined. Treatment with CLA resulted in lower levels of mammary tissue malondialdehyde (an end product of lipid peroxidation), but failed to change the levels of 8-hydroxydeoxyguanosine (a marker of oxidatively damaged DNA). Thus while CLA may have some antioxidant function *in vivo* in suppressing lipid peroxidation, its anticarcinogenic activity cannot be accounted for by protecting the target cell DNA against oxidative damage. The finding that the inhibitory effect of CLA maximized at 1% (regardless of the availability of linoleate in the diet) could conceivably point to a limiting step in the capacity to metabolize CLA to some active product(s) which is essential for cancer prevention.

Introduction

Conjugated linoleic acid (CLA*) is a positional and geometric isomer of linoleic acid (1). It is a minor fatty acid found preferentially in beef and dairy products (2). In contrast to linoleic acid, which has been found consistently to enhance mammary tumorigenesis in rodents over a wide concentration range (3–5), CLA expresses an inhibitory effect at levels of 1% or less in the diet (6,7). Recently, we described two distinct activities of CLA in mammary cancer prevention with the use of the methylnitrosourea (MNU) model (8). First, exposure to CLA during the early post-weaning and peripubertal period only (21–42 days of age) is sufficient to block subsequent tumorigenesis induced by a single dose of MNU given at 56 days of age. This observation suggests that CLA is able to effect certain changes in the immature mammary gland and render it less susceptible to neoplastic transformation later in life. Second, CLA is also active in suppressing tumor promotion/progression. However, this mode of action is different from the first in that once the mammary cells have been initiated by a carcinogen, a continuous intake of CLA is necessary to achieve maximal inhibition.

The above cited studies on CLA chemoprevention (6–8) were carried out in rats fed a 5% (w/w) fat diet formulated with corn oil. Currently, there is no information as to whether an increase in the level of fat or a substitution of the type of fat in the diet might affect the cancer inhibitory efficacy of CLA. The experiments described in this paper were designed to address this question. Because the anticancer agent of interest is a fatty acid, it is anticipated that the approach will provide some insight into its mechanism of action, depending on the outcome of these fat feeding experiments. For the fat level experiment, a custom formulated fat blend was used that simulates the fatty acid composition of the US diet. The idea was to examine the efficacy of CLA in the context of a fat consumption habit (10–20% by weight) that is relevant to humans. For the fat type experiment, a 20% (w/w) fat diet containing either corn oil (exclusively) or lard (predominantly) was used. Corn oil and lard differ significantly in their content of linoleate. Therefore, changes in the inhibitory activity of CLA in the presence of these two fat types may point to a possible interaction between CLA and linoleic acid in modulating tumor growth. Mammary cancer prevention by CLA under these various dietary conditions was evaluated using the rat dimethylbenz[*a*]anthracene (DMBA) model.

Previous work by Ha *et al.* (9) suggested that CLA is a potent antioxidant. At a molar ratio of 1 part CLA to 1000 parts linoleic acid, peroxide formation was reduced by >90% in a test tube assay. In fact, CLA was superior to tocopherol in this regard. In order to investigate whether interference with oxidative processes in cells might be implicated in cancer prevention by CLA, we examined the effect of CLA on two markers of cellular oxidative damage in the mammary tissue of rats fed either a high corn oil (unsaturated fat) or high lard (saturated fat) diet. These markers were malondialdehyde

*Abbreviations: CLA, conjugated linoleic acid; MNU, methylnitrosourea; DMBA, 7,12-dimethylbenz[*a*]anthracene; MDA, malondialdehyde; 8-OHdG, 8-hydroxydeoxyguanosine; TBA, thiobarbituric acid; AOS, antioxidant solution; dG, deoxyguanosine.

(MDA), an end product of lipid peroxidation, and 8-hydroxydeoxyguanosine (8-OHdG), an oxidized base isolated from DNA. Lipid peroxidation products have been implicated in mediating the formation of 8-OHdG in DNA (10). A recent publication from Thompson's laboratory has also reported that the number of 8-OHdG residues in mammary gland DNA increased in proportion to the degree of fatty acid unsaturation (as determined by iodine value) in the diet oils (11). More importantly, the rate of increase was sensitive to the presence or absence of nutritional levels of antioxidants such as vitamin E and selenium. Because of the above findings, we felt that these markers would be appropriate in assessing whether the antioxidant activity of CLA is manifest *in vivo*. Our goal was to investigate the possible relationship between the modulation of oxidative damage and the efficacy of cancer protection by CLA.

Materials and methods

Source and composition of CLA and other dietary fats

The method of CLA synthesis from >99% pure linoleic acid was detailed in our earlier publication (6). CLA was custom ordered from Nu-Chek Inc. (Elysian, MN). Gas chromatographic analysis showed that three particular isomers, *c*9,*t*11-, *t*9,*c*11- and *t*10,*c*12-CLA, constituted ~90% of the total. From our experience over several years, we have found that there were minimal variations in isomer distribution from batch to batch.

A 'vegetable fat blend' was prepared by Kraft Foods Inc. at their Technology Center (Glenview, IL). This fat blend was designed specifically to simulate the fatty acid composition in the average US diet. It consisted of 39.5% soybean oil, 22% palm oil, 12.5% high oleic sunflower oil, 9% cottonseed oil, 8.5% coconut oil and 8.5% cocoa butter. The reason that plant oils were used exclusively was to minimize the CLA content of the fat blend. Gas chromatographic analysis showed the following composition: C8:0, 0.9%; C10:0, 0.7%; C12:0, 5.1%; C14:0, 2.3%; C16:0, 18.8%; C16:1, 0.2%; C18:0, 5.6%; C18:1, 31.8%; C18:2, 29.5%; C18:3, 3.4%; C20:0, 0.4%; C22:0, 0.3%; CLA, not detectable. The above 'vegetable fat blend' has a polyunsaturate/monounsaturate/saturate fatty acid ratio of 1:1:1, which provided a fatty acid profile similar to that found in the typical US diet.

Two other commercial fats were used in this study: Mazola brand corn oil was obtained from Best Foods (Somerset, NJ) and lard was purchased from Harlan Teklad (Madison, WI). Lard contains ~0.3 mg CLA/g fat.

Design of mammary cancer chemoprevention experiments

Pathogen-free female Sprague–Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and housed in an environmentally controlled room with a 12 h light/12 h dark cycle. Mammary tumors were induced by a single i.g. dose of 7.5 mg DMBA at 50 days of age. Animals were palpated weekly to determine the time of appearance and location of tumors. At necropsy the mammary glands were exposed for the detection of non-palpable or microscopic tumors. Only histologically confirmed adenocarcinomas were reported in the results. In general between 10 and 15% of the tumors found in all groups (with or without CLA) were fibroadenomas. Tumor incidences at the final time point were compared by χ^2 analysis and the total tumor yield between groups was compared by frequency distribution analysis as described previously (12).

The first experiment involved feeding rats a diet containing different levels of the 'vegetable fat blend' at 10, 13.3, 16.7 and 20% by weight, with or without 1% CLA. Thus there were a total of eight dietary treatment groups in this design. All diets, which were prepared according to the AIN-76 formulation (6), were started 1 week before DMBA and continued until sacrifice (23 weeks post-DMBA). Ip has previously described the method of nutrient adjustment for diets containing different levels of fat so that the intake of protein, vitamins, minerals and calories was similar among the different groups (13).

At necropsy, the uninvolved (non-tumor-bearing) mammary glands from selected groups were excised and immediately dropped into liquid nitrogen. Upon removal from storage at −80°C, the frozen samples were pulverized and total fat was extracted with chloroform/methanol. The separation of phospholipids and neutral lipids was achieved with the use of a Sep-Pak silica cartridge as described in our earlier publication (6). Gas chromatographic analysis of the fatty acid methyl esters (including CLA) was determined by the method reported previously by Chin *et al.* (2).

The second experiment involved feeding a diet containing either 20% corn oil or a mixture of 8% corn oil + 12% lard, both with or without 1% CLA. Lard was chosen over tallow because of the much lower CLA content in lard (4 mg CLA/g fat in tallow versus 0.3 mg CLA/g fat in lard). The 12% lard in the diet therefore contributed <4 mg CLA/100 g diet, an amount that was insignificant compared with the level of 1% CLA used in this experiment. It should be noted that the lard diet also contained 8% corn oil. The reason was the previous finding of a high linoleate requirement for mammary tumorigenesis in the DMBA model (3,14). Similar to the above protocol, the feeding of the corn oil or lard diet ± 1% CLA was started 1 week before DMBA and continued until sacrifice.

The third experiment involved feeding a 20% corn oil diet with either 0.5, 1 or 1.5% CLA. The purpose was to determine the dose–response characteristics with respect to CLA in the presence of a linoleate-rich diet and to compare the results obtained here with our previous study of CLA efficacy (also at 0.5, 1 or 1.5%) in rats fed a 5% corn oil diet (6). Corn oil consists of ~60% linoleate. Thus the 5 and 20% corn oil diets contain ~3 and 12 g linoleate/100 g diet respectively. The purpose was to find out whether a diet that was rich in linoleate would require more CLA to achieve a maximal inhibitory effect.

Determination of MDA and 8-OHdG in mammary tissue

A separate experiment was set up to evaluate the effect of CLA on markers of lipid peroxidation and cellular oxidative damage in the mammary gland. Rats were fed the same corn oil or lard diet with or without 1% CLA as described in the above section. However, they were not treated with DMBA and the feeding period only lasted 2 months. At necropsy, the abdominal inguinal mammary gland chains (glands 4–6) were excised and dropped immediately into liquid nitrogen.

Tissue MDA was quantified as its thiobarbituric acid (TBA) derivative with reverse phase HPLC and photometric absorbance detection at 535 nm based on an extensive modification of the method described by Draper and Hadley (15). Mammary gland samples were homogenized with a Polytron in water containing a 1% antioxidant solution (AOS; 0.3 M dipyridyl and 2% butylated hydroxyanisole in ethanol), 1 part mammary tissue to 9 parts water (w/v). The samples were centrifuged at 6500 *g* and the fat plugs were removed, followed by further homogenization to resuspend the pellet. Since optimal reaction conditions were found to vary with protein concentration, an amount of homogenate containing ~1.2 mg protein was prepared for hydrolysis. The homogenate was combined with 7.5 µl 5 N HCl, 7.5 µl AOS and enough water to bring the volume to 1.5 ml. The covered tubes were heated to 96°C for 3 h. They were cooled quickly in tap water and 30 µl/tube sodium tungstate (Na_2WO_4) was added to facilitate precipitation of protein. After centrifugation at 6500 *g* for 10 min, 1 ml supernatant was then transferred to a clean glass tube. An aliquot of 0.75 ml TBA solution (1.11% TBA in 74 mM KOH) was added to each tube, followed by heating for 90 min to form the MDA–TBA adduct. Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. The MDA–TBA adduct was separated using a 4.6×150 mm C18 column (Beckman Ultrasphere ODS) and a mobile phase consisting of 32.5% methanol in 50 mM potassium phosphate buffer, pH 6.0, delivered at 1.5 ml/min. Photometric absorbance detection was at 535 nm. MDA was quantified by comparison of sample peak heights to those of the standard prepared from 1,1,3,3-tetramethoxypropane. The final results are expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (BioRad Protein Assay; BioRad Laboratories, Richmond, CA).

For the assay of 8-OHdG, the various procedures of DNA purification from the mammary gland, the enzymatic digestion of DNA to deoxynucleosides, the isocratic separation of 8-OHdG and deoxyguanosine (dG) by HPLC and the quantitation of 8-OHdG with an electrochemical detector were described in detail in a recent publication from Thompson's laboratory (11). The only modification introduced here was the elimination of phenol from the DNA isolation procedure. Detector response was linear from 10 to >800 pg/injection for 8-OHdG and from <500 to 6000 ng for dG. Results are reported as residues 8-OHdG/10^6 residues dG. The simultaneous analysis of both deoxynucleosides on a single HPLC injection abrogated the need for a recovery standard.

Results

Table I summarizes the mammary cancer chemoprevention data of CLA in rats fed different levels of fat. Tumor incidence at the time of necropsy was significantly reduced ($P < 0.05$) by CLA treatment in each of the four fat groups. In the absence of CLA supplementation, the total number of tumors increased by ~40% (from 71 to 98) in the range 10–20% dietary fat

Table I. Mammary cancer prevention by CLA in rats fed different levels of fat[a]

Dietary fat level	CLA	Tumor incidence	Total no. of tumors	Inhibition (%)[b]
10%		68.8%	71	
10%	1%	40.6%[c]	31[c]	56%
13.3%		81.3%	74	
13.3%	1%	46.9%[c]	40[c]	46%
16.7%		87.5%	94	
16.7%	1%	59.4%[c]	46[c]	51%
20%		90.6%	98	
20%	1%	59.4%[c]	49[c]	50%

[a]The fat used in this experiment was the 'vegetable fat blend' as described in Materials and methods. There were 32 rats/group.
[b]Percent inhibition was calculated using the tumor number data.
[c]$P < 0.05$ compared with the corresponding control group without CLA.

intake. However, as indicated in the last column of the table, the magnitude of tumor inhibition in CLA-treated rats was fairly consistent across all fat groups: 56% reduction on the 10% fat diet, 46% reduction on the 13.3% fat diet, 51% reduction on the 16.7% fat diet and 50% reduction on the 20% fat diet. This observation suggests that the efficacy of CLA in mammary cancer prevention is independent of the level of fat in the diet. The number of fibroadenomas found across all groups was very low and CLA did not affect the formation of these benign lesions.

The uninvolved (non-tumor-bearing) mammary glands of rats from selected groups were processed for fatty acid analysis in the neutral lipid and phospholipid fractions. The results from four dietary treatment groups (10 and 20% fat ± CLA) are presented in Table II. The data are expressed as percentages of total fatty acids. As indicated in footnote b, each value represents the mean of seven to eight samples, but since the standard error of the group mean is generally within 5% of the mean, the SEM is omitted from the table in order to make it more readable.

In the neutral lipid fraction, the three predominant fatty acids were C16:0, C18:1 and C18:2. In rats fed 10 and 20% fat without CLA the most significant change was an increase in C18:2 incorporation ($P < 0.05$) in the 20% fat group. The feeding of 1% CLA in diets containing 10 and 20% fat increased the netural lipid CLA content by 17.5-fold (from 0.2 to 3.5%) and 12-fold (from 0.2 to 2.4%) respectively, but did not alter the proportion of the other fatty acids in any substantial way.

Analysis of the phospholipid fraction showed that C16:0, C18:0, C18:2 and C20:4 accounted for >90% of total fatty acids. In particular, the high level of C20:4 incorporation was a distinctive characteristic of phospholipids. Thus the fatty acid profile found in phospholipids was different from that found in neutral lipids. Again there was no evidence that CLA supplementation perturbed the distribution of linoleate or other fatty acids in phospholipids. Interestingly, the increase in CLA incorporation in phospholipids (~0.3%) was much smaller in magnitude compared with that observed in neutral lipids (~2–3%). These findings suggest that there is selectivity of CLA incorporation in different classes of lipid.

Table III shows the mammary cancer chemopreventive activity of CLA in rats fed either an unsaturated fat (corn oil) or a saturated fat (lard) diet. It was apparent from the data that CLA was equally effective in suppressing tumorigenesis regardless of the type of dietary fat intake. Furthermore, the magnitude of tumor inhibition seen in this experiment was very similar to that described in the first experiment (Table I). In other words, with a constant dose of DMBA, feeding of 1% CLA reduced the number of mammary tumors by ~50% and this activity was evidently unaffected by the fat content (level or type) in the diet.

The efficacy of CLA in inhibiting lipid peroxidation and oxidative damage in mammary tissue was assessed by measuring MDA in mammary gland homogenate and 8-OHdG in mammary gland DNA. The results are presented in Table IV. In this experiment rats were fed the same corn oil or lard diet with or without 1% CLA as in the mammary carcinogenesis experiment shown in Table III. However, the animals were not treated with DMBA and they were sacrificed after 2 months of feeding. MDA levels were significantly elevated in rats fed the corn oil versus the lard diet ($P < 0.001$), this finding thus confirms the increased susceptibility of unsaturated fat to peroxidation. Feeding of CLA was associated with a reduction in MDA in the mammary tissue in both fat groups ($P < 0.001$). This effect was somewhat greater in rats fed a rich unsaturated fat diet (corn oil, 35% reduction; lard, 25% reduction; $P = 0.02$). Diet-associated differences in tissue levels of 8-OHdG were less remarkable. A 10–15% increase in 8-OHdG levels was detected with feeding the corn oil versus the lard diet ($P = 0.08$), however, tissue levels of this oxidized base were unaffected by CLA ($P = 0.42$).

It has been reported previously that 1% CLA produced a maximal inhibitory effect on mammary carcinogenesis in rats fed a 5% corn oil diet (6). No further protection was detected at levels above 1% CLA. In order to find out whether the dose–response characteristics with respect to CLA might be different in rats fed a 20% corn oil diet, an experiment was carried out to evaluate such a possibility (the protocol was otherwise identical to the previous 5% corn oil experiment with CLA supplemented at 0.5, 1 or 1.5%). As pointed out in Materials and methods, the difference in linoleate intake is substantial between a 5 and a 20% corn oil diet. If the action of CLA is totally dissociated from the availability of linoleic acid, the dose–response characteristics with respect to CLA are likely to be the same in rats consuming either a 5 or 20% corn oil diet. The results in Table V clearly show that maximal tumor inhibition was obtained with 1% CLA in rats fed a 20% corn oil diet. Increasing the concentration of CLA to 1.5% did not lead to a greater benefit in cancer protection.

Discussion

CLA is not the only fatty acid known to inhibit carcinogenesis. Eicosapentaenoic acid and docosahexaenoic acid, which are representative of the *n*-3 polyunsaturated fatty acids in fish oil, also fit this category (16). However, CLA differs from the fish oil fatty acids in two distinct aspects as far as their efficacies are concerned. Whereas fish oil is usually required at levels of ~10%, CLA at levels of 1% or less is sufficient to produce a significant cancer protective effect (7). Additionally, there are a number of papers which have indicated that an optimal ratio of fish oil to linoleate in the diet is critical in achieving maximal tumor inhibition (17–19). As can be seen from the present study, the potency of CLA in cancer prevention is largely dissociated from the quantity and type of dietary fats consumed by the host.

A possible mechanism of cancer prevention by fish oil *n*-3

Table II. CLA incorporation in neutral lipid and phospholipid fractions of mammary gland[a]

Fatty acid	Neutral lipid[b]				Phospholipid[b]			
	10% fat		20% fat		10% fat		20% fat	
	–CLA	+CLA	–CLA	+CLA	–CLA	+CLA	–CLA	+CLA
C12:0	1.2	1.2	1.6	1.6				
C14:0	1.7	1.9	1.7	1.6	1.1	1.1	1.2	1.1
C16:0	24.3	24.7	21.3	20.5	19.6	19.5	20.4	21.1
C16:1	3.9	3.9	3.0	1.5	0.5	0.4	0.4	0.3
C18:0	3.9	3.6	3.7	4.8	37.3	38.1	38.2	37.6
C18:1	42.3	38.9	40.4	38.8	5.1	4.9	4.6	4.8
C18:2	20.9	21.0	26.2	27.0	11.5	11.3	10.7	11.6
C18:3	0.9	0.9	1.3	1.2	0.5	0.4	0.4	0.3
C20:4	0.7	0.4	0.6	0.6	24.3	23.9	23.3	23.2
CLA	0.2	3.5	0.2	2.4	0.1	0.4	0.1	0.4
	100	100	100	100	100	100	100	100

[a]The samples were processed from uninvolved glands of rats reported in Table I.
[b]Results are expressed as percent of total fatty acids. The sum of each column is equal to 100%. Each value represents the mean of 7–8 samples, the SEM generally being within 5% of the mean.

Table III. Mammary cancer prevention by CLA in rats fed either an unsaturated fat or a saturated fat diet[a]

Dietary fat	CLA	Tumor incidence	Total no. of tumors	Inhibition (%)[b]
Corn oil		83.3%	68	
Corn oil	1%	40.0%[c]	35[c]	49%
Lard		80.0%	60	
Lard	1%	40.0%[c]	32[c]	47%

[a]The unsaturated fat diet contained 20% corn oil, while the saturated fat diet contained 8% corn oil + 12% lard. There were 30 rats per group.
[b]Percent inhibition was calculated using the tumor number data.
[c]$P < 0.05$ compared with the corresponding control group without CLA.

Table IV. Effect of CLA feeding on MDA and 8-OHdG levels in mammary gland[a,b]

Dietary fat	Malondialdehyde (nmol/mg protein)[c]		8-OHdG (residues/10^6 dG)[d]	
	–CLA	+CLA	–CLA	+CLA
Corn oil	1.39 ± 0.08	0.90 ± 0.14	4.00 ± 0.26	4.05 ± 0.20
Lard	0.43 ± 0.03	0.32 ± 0.02	3.38 ± 0.26	3.75 ± 0.30

[a]Rats were fed either the corn oil or lard diet with or without 1% CLA for 2 months.
[b]Results are expressed as mean ± SE ($n = 9$).
[c]By factorial analyses of variance the following effects on malondialdehyde were noted. Type of fat, F ratio 88.903, $P < 0.001$; CLA, F ratio 13.76, $P = 0.001$; interaction between fat type and CLA, F ratio 5.62, $P = 0.024$.
[d]By factorial analyses of variance the following effects on 8-OHdG were noted. Type of fat, F ratio 3.18, $P = 0.08$; CLA, F ratio 0.42, $P = 0.42$; interaction between fat type and CLA, F ratio 0.37, $P = 0.54$.

polyunsaturated fatty acids has been postulated to be through perturbation of eicosanoid biosynthesis (19,20). *In vivo*, linoleic acid is converted to arachidonic acid, which is the precursor for the various eicosanoids produced via either the cyclo-oxygenase or lipoxygenase pathways. The data presented in this paper tend to suggest that CLA is unlikely to interfere with the metabolic cascade involved in converting linoleic acid to eicosanoids. First, the anticarcinogenic efficacy of CLA was not affected by variations in linoleate intake, as demonstrated by the experiments reported in Tables I and III.

Table V. Mammary cancer prevention by different levels of CLA in rats fed a 20% corn oil diet[a]

CLA	Tumor incidence	Total no. of tumors	Inhibition (%)[b]
	93.3%	87	
0.5%	70.0%	53[c]	39%
1%	50.0%[c]	37[c]	57%
1.5%	46.7%[c]	34[c]	61%

[a]There were 30 rats per group.
[b]Percent inhibition was calculated using the tumor number data.
[c]$P < 0.05$ compared with the control group without CLA.

Second, similar dose–response characteristics with respect to CLA at 1% and below were noted in rats fed either a 5 or 20% corn oil diet (6; Table V). No further protection was evident with supplementation of CLA above 1% in both cases. The fact that the effect of CLA maximizes at 1% may indicate a limiting step in the capacity to metabolize CLA to some active product(s) which is essential for inhibition of carcinogenesis. Suffice it to note that absorption of CLA is probably not a confounding factor here, because tissue accumulation of CLA continues to rise with dietary levels above 1% (unpublished data).

In all the carcinogenesis experiments included in this paper, CLA was given to the animals starting 1 week before DMBA and continuing until termination of the experiment. We adopted this protocol initially with the experiment shown in Table I, and in order to maintain uniformity, followed the same protocol in subsequent experiments reported in Tables III and V. However, we have observed that CLA does not affect DMBA binding to mammary cell DNA (7) nor does it affect phase II conjugating enzymes, such as glutathione S-transferase and UDP-glucuronyl transferase (6). In other words, CLA is expected to have little influence on DMBA activation or detoxification. It can thus be conjectured that the major impact of CLA on mammary carcinogenesis with the above protocol is due to its inhibitory effect on tumor promotion or progression.

Some explanation is called for here about the finding that in rats which were maintained on the 'vegetable fat blend' diet there was a small but detectable amount of CLA in the mammary tissue even though the animals did not receive an

exogenous supply of CLA. In an attempt to determine whether the bacterial flora in the colon of rats could be the source of CLA, Chin *et al.* (21) have recently examined the tissue levels of CLA between conventional and germ-free rats which were fed diets with or without free linoleic acid. With the conventional rats, tissue CLA concentrations were 5–10 times higher in those animals given a 5% linoleic acid supplement. In contrast, CLA concentrations in tissues of germ-free rats were not affected by the addition of linoleic acid. These findings strongly suggest that the intestinal bacterial flora of rats is capable of converting linoleic acid to CLA.

As shown by the data in Table II, there might be some selectivity in the incorporation of CLA into different lipids following ingestion of a diet rich in CLA. When expressed as a percentage of total fatty acids, CLA is more abundant in neutral lipids than in phospholipids. It is unclear whether this uneven distribution of CLA in various lipid fractions has any relevance to cancer risk modulation. Because of the configuration of the *trans* double bond(s) in CLA, the incorporation of CLA in membrane phospholipids could conceivably diminish the fluidity of the lipid bilayer. On the other hand, the small amount of CLA in phospholipids tends to argue against the significance of a membrane effect. The storage of CLA in neutral lipids could portend the importance of this pool in providing a continuous supply of CLA for generation of some active metabolite(s). Further research is needed to examine the rate of turnover of CLA in neutral lipids and the possible oxidative modification of CLA, similar to that observed with linoleic acid (22–24).

The ability of CLA to suppress lipid peroxidation was first described by Pariza's laboratory (9). In that work linoleic acid was exposed to air and moderate heat with or without a very small amount of CLA for an extended period of time. Under those conditions the degree of linoleic acid oxidation (peroxide value) was determined by the thiocyanate method (25). It was hypothesized that an oxidized derivative of CLA might be the active antioxidant species, rather than CLA itself (9). According to the proposed scheme, which is supported by spectrophotometric evidence, a β-hydroxy acrolein moiety would be introduced across the conjugated double bond of CLA following reaction with a hydroxyl or peroxyl radical and molecular oxygen. Antioxidant activity would result from chelation of iron by the β-hydroxy acrolein functional group, thereby interfering with the Fenton reaction. A recent paper by van den Berg (26), however, contradicted the above conclusion. These investigators studied whether CLA could protect membrane vesicles composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine from oxidative modification under various conditions. Oxidation was determined by direct spectrophotometric measurement of conjugated diene formation and by gas chromatographic/mass spectrometric analysis of fatty acids. It was found that CLA neither acts as a radical scavenger nor is it converted into a metal chelator in the Fe^{2+} ion-dependent oxidative reaction. Thus, at least in a model membrane system, CLA does not function as an effective antioxidant or antioxidant precursor.

The results presented in Table IV may provide new clues as to the effect of CLA on oxidative events *in vivo*. MDA levels were lower in mammary tissue of CLA-treated rats and the suppressive effect was somewhat greater in rats fed the more unsaturated dietary fat. Since MDA was measured in whole mammary gland homogenate, it is likely to represent the peroxidation of neutral lipids, which are found predominantly in the mammary gland adipocytes. As shown in Table II, CLA is also preferentially incorporated in the neutral lipid fraction. On the other hand, the levels of 8-OHdG, which are only marginally affected by the type of dietary fat and not at all by CLA supplementation, are probably a better indicator of DNA oxidative damage that may be causally related to tumor promotion/progression. The presence of 8-OHdG has been implicated in mismatching errors and base substitutions in DNA replication (27,28). The absence of a detectable effect of CLA on 8-OHdG is also consistent with the lack of a significant accumulation of CLA in the phospholipid fraction, which is likely to originate from mammary epithelial cells. In summary, based on the information obtained in this study, we believe that the ability of CLA to inhibit mammary carcinogenesis is not mediated by protecting the target cell DNA against damage induced by reactive oxygen species. Current research is focused on using a mammary epithelial cell culture model (29,30) to generate new insights into potential mechanisms of CLA in regulating growth and differentiation.

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Retention of conjugated linoleic acid in the mammary gland is associated with tumor inhibition during the post-initiation phase of carcinogenesis

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Conjugated linoleic acid (CLA) has been reported to have significant activity in inhibiting mammary carcinogenesis. A major objective of this study was to evaluate how changes in the concentration of CLA in mammary tissue as a function of CLA exposure/withdrawal were correlated with the rate of occurrence of mammary carcinomas. Rats treated with a single dose of dimethylbenz[*a*]anthracene (DMBA) at 50 days of age were given 1% CLA in the diet for either 4 weeks, 8 weeks or continuously following carcinogen administration. No cancer protection was evident in the 4 or 8 week-CLA treatment groups. Significant tumor inhibition was observed only in rats that were given CLA for the entire duration of the experiment (20 weeks). Analysis of CLA in the mammary gland showed that the incorporation of CLA was much higher in neutral lipids than in phospholipids. When CLA was removed from the diet, neutral lipid- and phospholipid-CLA returned to basal values in about 4 and 8 weeks, respectively. The rate of disappearance of neutral lipid-CLA (rather than phospholipid-CLA) subsequent to CLA withdrawal paralleled more closely the rate of occurrence of new tumors in the target tissue. It appears that neutral lipid-CLA may be a more sensitive marker of tumor protection than phospholipid-CLA. However, the physiological relevance of CLA accumulation in mammary lipids is unclear and remains to be determined. A secondary goal of this study was to investigate whether CLA might selectively inhibit clonal expansion of DMBA-initiated mammary epithelial cells with wild-type versus codon 61 mutated *Ha-ras* genes. Approximately 16% of carcinomas in the control group (without CLA) were found to express codon 61 *ras* mutation. Although continuous treatment with CLA reduced the total number of carcinomas by 70%, it did not alter the proportion of *ras* mutant versus wild-type carcinomas, suggesting that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of the *ras* mutation.

Introduction

Conjugated linoleic acid (CLA*) is a minor fatty acid found preferentially in red meat and dairy products (1). The biosynthesis of CLA in ruminants is accounted for by a rumen bacterium, which is known to convert linoleic acid to stearic acid via CLA (2). Over the past decade, research from several laboratories has shown that CLA expresses powerful activity in cancer protection in a number of animal models (3–7). Feeding diets containing ≤1% CLA results in a dose-dependent suppression of tumor development in the mammary gland (8). CLA appears to have a dual effect in the modulation of mammary carcinogenesis in rats. First, exposure to CLA during the window of active mammary gland morphogenesis may reduce the proliferation of epithelial end bud cells, thus conceivably rendering the target cell population less susceptible to carcinogen-induced neoplastic transformation (8,9). Second, CLA is also capable of inhibiting tumor promotion/progression (9); however, a continuous supply of CLA is required for this mechanism of action.

The above study regarding the effectiveness of CLA in blocking tumor progression was carried out in the methylnitrosourea (MNU)-induced mammary carcinogenesis model in rats fed a 5% corn oil diet (9). One objective of the experiments reported in this study was to confirm the necessity of maintaining CLA intake after cancer induction by using dimethylbenz[*a*]anthracene (DMBA)-treated rats fed a 20% corn oil diet. It was considered important to assess whether the requirement for continuous CLA feeding was dependent on the nature of the carcinogen and the fat content of the diet. Rats were therefore given CLA for a duration of either 4, 8 or 20 weeks, starting immediately after a single dose of DMBA, to evaluate the anti-carcinogenic efficacy of these various intervention regimens. The kinetics of mammary tissue CLA retention as a function of CLA exposure/withdrawal was also analyzed in order to determine the correlation between time-dependent changes in tissue concentrations of CLA and effectiveness of cancer protection.

Additionally, we were interested in finding out whether CLA might selectively inhibit the clonal expansion of DMBA-initiated cells carrying either the wild type or codon 61 mutated *Ha-ras* gene. Previous work from Thompson's laboratory has shown that high dietary levels of linoleic acid preferentially increased the number of wild type *Ha-ras* mammary tumors, but not the codon 12 mutant *Ha-ras* tumors, in the rat MNU model (10). In chemical carcinogenesis, specific *ras* mutations are induced and are believed to be involved in early stages of tumor development (11–14). Generally, *ras* mutation is considered to be permissive but not sufficient for carcinogenesis. Thus the *ras* genotype was used as a marker in the present study to identify subpopulations of neoplastically transformed cells that might be differentially modulated by CLA intervention.

Materials and methods

Pathogen-free female Sprague–Dawley rats were purchased from Charles River Breeding Laboratories at 45 days of age. They were fed a 20% corn oil diet (6) and were intubated with a single dose of 10 mg of DMBA at 50 days of age for the induction of mammary tumors. Supplementation of CLA (Nu-Chek, Elysian, MN) at 1% in the diet was started 4 days after carcinogen administration. A total of 90 rats were given CLA and were divided equally

*Abbreviations: CLA, conjugated linoleic acid; DMBA, dimethylbenz[*a*]anthracene; MNU, methylnitrosourea; PCR/RFLP, polymerase chain reaction-generated restriction fragment length polymorphism.

Table I. Time course of wild-type and mutant *ras* mammary tumor appearance in control and CLA-supplemented rats[a]

Treatment	*ras* Genotype	Total (%)
Control	Wild-type	49 (84%)
	Mutant	9 (16%)
4 weeks-CLA	Wild-type	49 (92%)
	Mutant	4 (8%)
8 weeks-CLA	Wild-type	43 (86%)
	Mutant	7 (14%)
Continuous-CLA	Wild-type	13 (81%)
	Mutant	3 (19%)

[a]These tumors were harvested from the mammary carcinogenesis experiment described in Figure 1.

into three groups according to the length of CLA treatment: 4 weeks, 8 weeks or continuously until the end of the experiment. Control rats (n = 30) were not given CLA at any time during the study.

Animals were palpated weekly for mammary tumors; the time of appearance and location of tumors in the mammary gland were recorded. The experiment was terminated 20 weeks after DMBA. By that time, the development of palpable tumors had plateaued for several weeks across all groups. Only histologically confirmed adenocarcinomas were reported in the results. Tumor incidences at the final time point were compared by chi squared analysis, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis as described previously (15).

A total of 177 mammary adenocarcinomas were harvested from the above carcinogenesis bioassay. They were individually identified after excision so that each one could be tracked to its time of appearance in a particular rat. All 177 paraffin block-embedded tumors were analyzed for codon 61 *ras* mutation (CAA→CTA) by a modification of the polymerase chain reaction-generated restriction fragment length polymorphism (PCR/RFLP) method as described by Kumar and Barbacid (16). Two 5-μ sections were prepared side-by-side from the same paraffin block, one mounted on a plastic slide, the other on a glass slide, which was subsequently stained with hematoxylin and eosin for the identification of tumor cell foci under the microscope. The exact same area of interest was matched on the plastic slide and was then cut out for DNA extraction (17). The primers used for PCR amplification were 5′-GAGACGTGTTTACTGGACATCTT-3′ and 5′-GTGTTGTTGATGGCAAA-TACACAGAGG-3′ (synthesized by Integrated DNA Technologies, Coralville, IA), which yielded a 116 bp PCR product (18,19). The PCR reaction mixture contained 5 μl of DNA extract, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 15 μM deoxynucleotide triphosphate, 1 μCi of α-[^{32}P]dCTP, 0.1 μM upstream and downstream primers, and 0.5 units AmpliTaq DNA polymerase (Perkin–Elmer, Norwalk, CT). For each batch of PCR reaction, PCR-grade H_2O was used as a blank, DNA from a tumor bearing *Ha-ras* codon 61 mutation as a positive control, and DNA from normal mammary gland as a negative control. Amplification was carried out for 40 cycles at: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min using a GeneAmp PCR system 9600 (Perkin–Elmer). The codon 61 A→T mutation introduces a Xba I restriction site into the 116 bp PCR product, which upon digestion, generates two fragments of 80 and 36 bp that are diagnostic for the mutation. In contrast, the PCR product of the normal gene contains a sequence that is not susceptible to digestion by Xba I. The digested materials were separated by electrophoresis on a 6% polyacrylamide gel, and detected by autoradiography on X-ray film.

To study the kinetics of CLA retention in the mammary gland, a two-part experiment was conducted to examine (i) the rate of increase of tissue CLA following the start of CLA feeding, and (ii) the rate of disappearance of tissue CLA following CLA withdrawal. For the first part, 60-day-old rats (age-matched to those in the above carcinogenesis experiment but not given DMBA) were fed a 1% CLA diet and were killed at 1, 2, 4, 6 or 8 weeks later. For the second part, rats were fed a 1% CLA diet for 8 weeks, the treatment was discontinued and necropsy was timed at 1, 2, 4 or 6 weeks after CLA withdrawal. Total lipid was extracted from the mammary gland by chloroform/methanol. The separation of neutral lipids and phospholipids was achieved with the use of a Sep-Pak silica cartridge as described in an earlier publication (5). Gas chromatographic analysis of the CLA methyl ester was determined by the method reported previously by Chin *et al.* (1).

Results

Figure 1 shows the time course of mammary tumor development in control rats or rats fed CLA for various lengths of

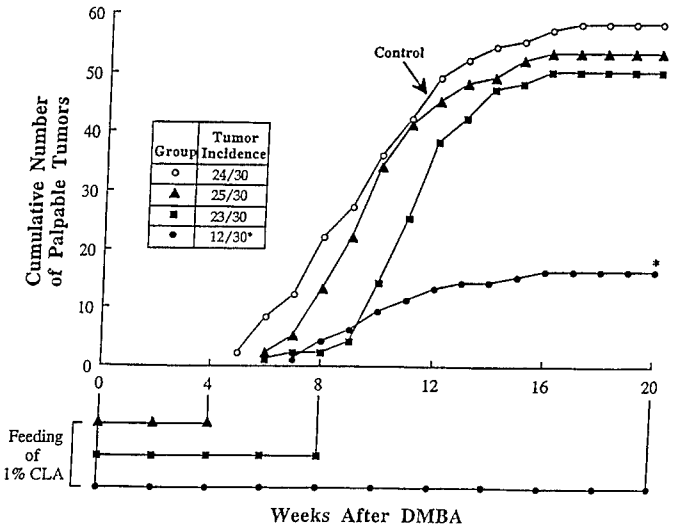

Fig. 1. Effect of interrupted versus continuous CLA feeding after DMBA administration on mammary carcinogenesis. The duration of CLA feeding in the three supplemented groups is indicated along the x-axis time line by the filled symbols, which match the time course of mammary tumor development on the main body of the diagram. Control group without CLA supplementation is represented by the open circle. The asterisk denotes statistically significant difference ($P < 0.05$) from the control data.

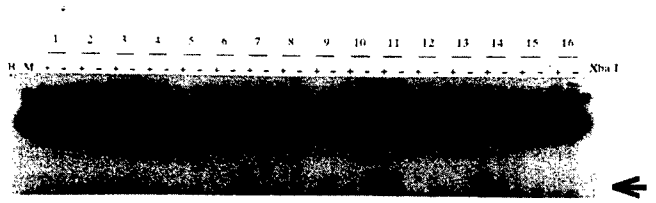

Fig. 2. Detection of *Ha-ras* codon 61 CAA→CTA mutation by PCR/RFLP method. The mutation produces a XbaI site in amplified 116-bp DNA fragment. Upon separation of the XbaI-digested product in 6% polyacrylamide gel, the presence of a 80-bp band (arrow) serves as a diagnostic marker for the mutation. PCR products were labeled with tracer amount of α-[^{32}P]dCTP and detected by autoradiography. Lane B, blank without template DNA; lane M, normal mammary gland DNA as a negative control; lane 1–16, mammary adenocarcinomas DNA. + and –, 5 μl of PCR product treated with or without 5 units of XbaI, respectively.

time. It can be seen that short-term feeding of CLA for only 4 or 8 weeks after DMBA administration was not effective in tumor inhibition. In the 8 week-CLA treatment group, the time course curve was shifted slightly to the right, suggesting a delay of about 2 to 3 weeks in the appearance of tumors. However, as soon as CLA was withdrawn, the rate of tumor appearance resumed at a rapid pace. At the time of necropsy, the difference in tumor occurrence between the control group and the 8 week-CLA treatment group was not statistically significant. In contrast, marked cancer protection, as judged by a 50% reduction in tumor incidence and a 70% reduction in the total number of tumors, was observed in rats that were given CLA for the entire duration of the study.

Figure 2 shows some representative electrophoresis autoradiograms of XbaI digested PCR products from tumors with either the wild-type or codon 61 mutant *ras* gene. The arrow in the diagram indicates the presence of a 80-bp band, which is diagnostic for the mutation. Table I summarizes the frequency distribution of both wild-type and mutant *ras* mammary

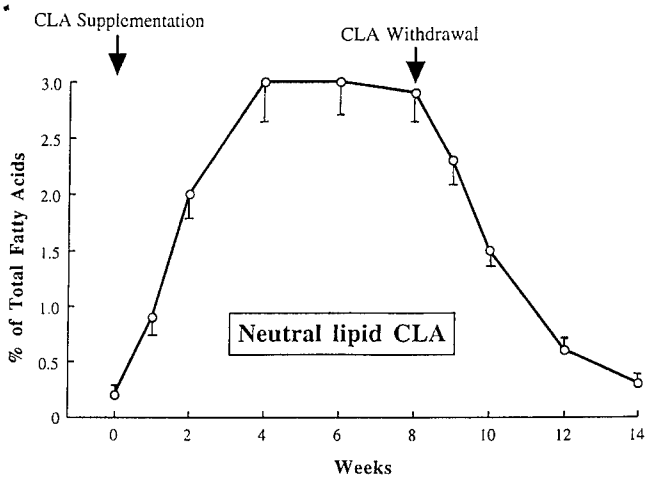

Fig. 3. The kinetics of CLA retention in neutral lipids of mammary gland following CLA supplementation and withdrawal. The results are expressed as the percentage of total fatty acids, mean ± SE (n = 6).

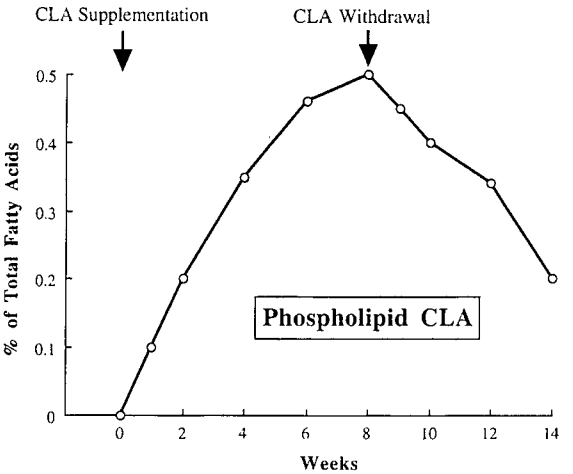

Fig. 4. The kinetics of CLA retention in phospholipids of mammary gland following CLA supplementation and withdrawal.

carcinomas from the above experiment. In the control group, 16% of the tumors expressed the mutant *ras* gene. Continuous feeding of CLA reduced the total number of carcinomas by 70%, but was found to suppress approximately the same proportion of wild-type and mutant *ras* carcinomas in comparison with the control group. Short-term treatment with CLA for 4 or 8 weeks did not decrease significantly the total number of carcinomas, nor did it alter markedly the distribution of carcinomas carrying either the wild-type or mutant *ras* gene. Overall, there was no unusual pattern in the time of appearance of the *ras* mutant tumors due to CLA intervention (data not shown). Thus our results indicate that CLA inhibited carcinogenesis irrespective of the presence or absence of the codon 61 *ras* mutation.

Figure 3 shows the rates of CLA accumulation and disappearance in the neutral lipid fraction of the mammary tissue following CLA administration and withdrawal. As the results indicate, the incorporation was rapid once CLA was added to the animals' diets. The level reached ~70% of maximum after 2 weeks of feeding, and plateaued after 4 weeks. At the peak, CLA was present at roughly 3% of total fatty acids in the neutral lipid fraction. In this experiment, some animals were given CLA for 8 weeks. The regimen was stopped, and the decrease in CLA concentration was then plotted in the same composite diagram. Figure 3 shows that as soon as CLA was discontinued, the rate of disappearance from the mammary tissue was equally fast, with a return to basal value in about 4 weeks.

Figure 4 shows the increases and decreases of mammary gland phospholipid CLA from the same experiment. It should be noted that during CLA supplementation, the concentration of CLA in phospholipids (expressed as percent of total fatty acids) was, on the average, an order of magnitude lower than the concentration in neutral lipids. Interestingly, the rate of change of phospholipid CLA in either the upswing or downswing of the exposure/withdrawal curve was slower compared with that observed with neutral lipid CLA. After the start of CLA feeding, the maximum level in phospholipids was not attained until about 6 to 8 weeks later. Similarly, a diminished but still detectable amount of CLA was present by 6 weeks subsequent to the removal of CLA from the diet.

Discussion

The present study confirms our previous report that a continuous supply of CLA is necessary for maximum tumor inhibition in the post-initiation phase of mammary carcinogenesis. As pointed out in the Introduction, the first experiment was done in MNU-treated rats fed a 5% corn oil diet (9), whereas the repeat experiment described here was carried out in DMBA-treated rats fed a 20% corn oil diet. Thus this characteristic of CLA in chemoprevention is apparently not dependent on specific genomic mutation induced at the time of initiation or the availability of linoleic acid fed to the animals during tumor progression. It might be instructive to contrast the effects of CLA and linoleic acid at this point. Our study here indicated that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of *ras* mutation. Linoleic acid, on the other hand, has been demonstrated to promote selectively the development of the wild type *ras* tumors, but not the mutant *ras* tumors, in MNU-treated rats (10). Recent data also suggested that the response to CLA is unlikely to be due to a displacement of linoleic acid in the mammary tissue (20). Collectively, the above information provides supportive evidence that these two fatty acids may have distinctive mechanisms in the modulation of mammary carcinogenesis.

Mutations of the *ras* gene have been reported to occur in a target organ- and chemical carcinogen-specific manner in a number of experimental models (21). Zarbl *et al.* (22) have previously described that in MNU-induced mammary tumors, GGA→GAA mutation in codon 12 of the *Ha-ras* proto-oncogene is a common event. The mutation probably results from methylation of guanine by diazomethane, a spontaneous decomposition product of MNU. In contrast, these same investigators found that only 21% (three out of 14) of DMBA-induced mammary tumors express a CAA→CTA mutation in codon 61 of the *Ha-ras* gene (22). It has been proposed that the A→T transversion is probably due to the affinity of the DMBA diol epoxide to the adenine residue as well as to the sequence selectivity in binding of the metabolite to the *Ha-ras* DNA (23,24). To our knowledge, there has been one other study examining *Ha-ras* codon 61 mutation in the DMBA model. Interestingly, Waldmann *et al.* (25) did not find such a mutation in a total of 50 tumors. The relatively low incidence of *Ha-ras* codon 61 mutation in our study is similar to that reported by Zarbl *et al.* (22). However, it should be noted that

our analysis was performed on a much larger sample size. In any case, the data in Table I clearly indicate that DMBA-initiated cells, with or without a *Ha-ras* codon 61 mutation, are equally sensitive to the inhibitory activity of CLA.

Recent studies by Banni *et al.* (26) have shown that in rats fed only 0.04% CLA in the diet for 1 week, conjugated diene-C18:3 and -C20:3 were recovered in the liver. Thus it appears that desaturation and elongation of CLA can occur *in vivo* while maintaining the conjugated diene structure. The presence of a conjugated diene-C20:4 metabolite could compete with arachidonic acid for the cyclo-oxygenase and lipo-oxygenase enzymes, thereby altering the biosynthesis of prostaglandins, thromboxanes and leucotrienes. These downstream products of arachadonic acid have been implicated by many investigators to be associated with promotion of carcinogenesis (27–32). By acting as a precursor to conjugated diene-C20:4, CLA could potentially play the role of a metabolic modulator in this process. Thus it becomes imperative to determine if conjugated diene-C20:4 is found in the mammary gland and if it is compartmentalized in a specific lipid fraction.

From our CLA analytical data, it is tempting to postulate that neutral lipid CLA may be a better indicator of protection than phospholipid CLA. Neutral lipid is far more plentiful than phospholipid in the mammary gland (see our previous work quoted in Reference 6). The larger pool of CLA in the former fraction may be more responsive to dietary intake because it serves as a depot for fatty acids that are not immediately utilized. Furthermore, the rate of decay of neutral lipid CLA following CLA withdrawal (Figure 3) seems to match more closely the rate of emergence of new tumors (refer to 4 week- or 8 week-CLA groups in Figure 1).

At first glance, the data on the changes in tissue concentration of CLA (Figures 3 and 4) appear to provide a reasonable explanation of why an uninterrupted supply of CLA is necessary to achieve tumor inhibition. As long as there is an abundant source of CLA present in the target organ, tumor appearance will be blocked or delayed. However, one must not lose sight of the possibility that CLA or a metabolite may induce an effect that is independent of its accumulation in mammary lipids. Future research will be aimed at delineating: (i) whether neutral lipid and phospholipid CLA levels simply represent indicators of CLA exposure; (ii) whether they serve as a local supply of CLA for further metabolism; and (iii) whether different cellular compartments of the mammary gland are involved in the accumulation and metabolism of CLA that ultimately leads to cancer prevention.

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Morphological and Biochemical Status of the Mammary Gland as Influenced by Conjugated Linoleic Acid: Implication for a Reduction in Mammary Cancer Risk[1]

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ABSTRACT

Previous research showed that treatment with conjugated linoleic acid (CLA) during the period of active mammary gland morphogenesis was sufficient to confer a lasting protection against subsequent mammary tumorigenesis induced by methylnitrosourea. The present study was designed to characterize certain morphological and biochemical changes of the mammary gland that might potentially render it less susceptible to cancer induction. Female Sprague Dawley rats were fed a 1% CLA diet from weaning until about 50 days of age. The mammary gland parameters under investigation included (*a*) the deposition of neutral lipid, (*b*) the identification and quantification of CLA and its metabolites, (*c*) the density of the epithelium, and (*d*) the proliferative activity of various structural components. Our results showed that CLA treatment did not affect total fat deposition in the mammary tissue nor the extent of epithelial invasion into the surrounding fat pad but was able to cause a 20% reduction in the density of the ductal-lobular tree as determined by digitized image analysis of the whole mounts. This was accompanied by a suppression of bromodeoxyuridine labeling in the terminal end buds and lobuloalveolar buds. The recovery of desaturation and elongation products of CLA in the mammary gland confirmed our prior suggestion that the metabolism of CLA might be critical to risk modulation. The significance of the above findings was investigated in a mammary carcinogenesis bioassay with the use of the dimethylbenz[*a*]anthracene model. When CLA was started at weaning and continued for 6 months until the end of the experiment, this schedule of supplementation produced essentially the same magnitude of mammary tumor inhibition in the dimethylbenz[*a*]anthracene model as that produced by 1 month of CLA feeding from weaning. The observation is consistent with the hypothesis that exposure to CLA during the time of mammary gland maturation may modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation.

INTRODUCTION

Past research showed that CLA[3] has powerful cancer protective activity in a number of animal tumor models (1–6). With respect to mammary carcinogenesis in the rat, dietary supplementation of CLA has been reported to exert a unique inhibitory effect that is not commonly shared by many anticancer agents. The recent work of Ip and coworkers (7) demonstrated that CLA exposure limited to the period of active mammary gland development was sufficient to confer a lasting protection against subsequent chemically induced tumorigenesis in the target organ. In that experiment, CLA was fed to the animals between 21 days (weaning) and 55 days of age, and a single dose of MNU was administered for mammary tumor induction at day 56. No CLA was provided in the diet after carcinogen treatment. The above observation has a very significant implication for cancer prevention and is the subject of additional research reported herein.

The present study was designed to examine certain morphological and biochemical changes of the mammary gland after 1 month of CLA supplementation starting from weaning. Specifically, our objective was to define the degree of morphological development of the mammary gland as well as alteration in its biochemical constituents that might potentially render it less susceptible to cancer risk. The end points under investigation included (*a*) the total amount of lipid in the mammary gland, (*b*) the identification and quantification of CLA and its metabolites in the mammary tissue, (*c*) the density of the mammary epithelium and the area of the mammary fat pad occupied by the mammary tree, and (*d*) the proliferative activity of various mammary structural components.

Different carcinogens are known to cause specific mutations that may contribute to the process of oncogenesis (8). As indicated earlier, our initial study regarding the protective effect of CLA following short-term supplementation (instituted at an early age and prior to carcinogen treatment) was done using the MNU model. To rule out that this phenomenon of risk reduction is not an occurrence that is only characteristic of MNU-induced oncomutations, we repeated the tumor experiment with the DMBA model in the study reported here. Additionally, we also evaluated the relative efficacy of the timing of CLA supplementation by comparing the magnitude of mammary cancer inhibition in rats that were fed CLA from weaning to 50 days of age *versus* those that were given CLA from weaning to the end of the experiment, *i.e.*, including the entire period of tumor promotion and progression. This kind of information is important not only for formulating prevention strategies but also for targeting future research directions.

MATERIALS AND METHODS

Animals and CLA Supplementation. Pathogen-free female Sprague Dawley rats were purchased from Charles River Breeding Laboratories at weaning. They were fed the basal AIN-76A diet with or without supplementation with 1% CLA (Nu-Chek, Elysian, MN). For the studies that were designed to examine the morphological and biochemical changes of the mammary gland, the animals were sacrificed after 1 month on either the control or the CLA diet. The number of rats used in each type of analysis is indicated in the "Results" section. For the mammary carcinogenesis experiment, a total of 120 rats were divided equally into four groups according to the following dietary treatment: group A, control diet from weaning to termination of the experiment (see below); group B, 1% CLA diet from weaning to 50 days of age followed by a switch to the control diet; group C, 1% CLA diet from 55 days of age to termination of the experiment; and group D, 1% CLA diet from weaning to termination of the experiment.

Lipid Extraction. Total lipid was extracted from frozen pulverized mammary tissue by the method of Folch *et al.* (9). Neutral lipid and phospholipid were separated with the use of a Sep-Pak silica cartridge, as described in an earlier publication (5). The amount of lipid recovered in each of these fractions was measured.

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[3] The abbreviations used are: CLA, conjugated linoleic acid; MNU, methylnitrosourea; DMBA, dimethylbenz[*a*]anthracene; BrdUrd, bromodeoxyuridine.

Quantification of Conjugated Diene Polyunsaturated Fatty Acids. For the determination of conjugated diene polyunsaturated fatty acids, total lipid extracted from the abdominal-inguinal mammary glands (without separation into neutral lipid and phospholipid) was used as the starting material. Free fatty acids were obtained by a mild saponification procedure as described by Banni *et al.* (10) and collected in *n*-hexane. After solvent evaporation, the residue was redissolved in CH_3CN/0.14% CH_3COOH (v/v) for injection into the high-performance liquid chromatography system. Separation of unsaturated fatty acids was carried out with a Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector 1040M (Hewlett-Packard, Palo Alto, CA). A C-18 Alltech Adsorbosphere column, 5-μm particle size, 250 × 4.6 mm, was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min. Nonconjugated diene unsaturated fatty acids were detected at 200 nm, and conjugated diene unsaturated fatty acids were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.3 s and were stored electronically. Second-derivative UV spectra of the conjugated diene fatty acids were generated using the Phoenix 3D HP Chemstation software. These spectra were taken to confirm the identification of the high-performance liquid chromatography peaks. Details of the methodology regarding the characterization of conjugated diene fatty acids in both reference and biological samples have recently been published by Banni and coworkers (11).

Preparation of Mammary Gland Whole Mount and Analysis of Epithelial Density. The abdominal-inguinal mammary gland chain was excised in one piece and stretched onto a 75 × 50 mm microscope slide. The whole mount was fixed in 10% buffered formalin for 12–18 h and rinsed in distilled water. It was then dehydrated using a series of ethanol solutions (70, 95, and 100%) for 1 h each and cleared with two changes of toluene for 1 h each. The tissue was rehydrated with water and immersed in alum carmine stain for 5–7 days. Once staining was complete, the whole mount was dehydrated using ethanol as described above and cleared with one change of xylene for 2 h. Each whole mount was then placed in a 4 × 6-inch heat-sealable pouch and filled with 20 ml of methyl salicylate. Methyl salicylate was chosen as the clearing agent, because its refractory index is very close to that of tissue. This resulted in superior photographic resolution with a clean background. The pouch was left overnight, and on the next day, it was pressed flat to remove excess methyl salicylate and air. All whole mounts were photographed using a Nikon 55 mm camera equipped with a digital camera back. The digitized images were analyzed by scanning densitometry.

All images were presented as an array of pixels. The manipulation of images and all calculations of the parameters were performed on an MPC 200 workstation running the UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT). For each mammary gland chain, the same anatomical region was assessed. This assessment was restricted to the abdominal inguinal mammary gland chain and specifically to the region cephalic to the anterior-most lymph node located in the fourth mammary gland. This lymph node served as an anatomical landmark from which a line perpendicular to the cephalocaudal axis of the animal was projected across the image of the mammary gland. The gland cephalic to this line was cut into image segments, including only the area occupied by the mammary epithelium. The absorbance of each segment and the total area of each segment were then quantified and saved to disk. The density and area of all segments were then summed and averaged. An interactive density thresholding technique was used to select the pixel intensity value (gray value) above which pixels were discriminated from the remainder of the gland as mammary epithelium. This was done for all images by one observer. Preliminary studies (data not shown) indicated that the use of absorbance per unit area is capable of detecting differences in the maturity of the mammary gland that occurs in young rats as well as the remarkable differences in gland development observed between the virgin and pregnant states. The above approach is based on previous work in rats and mice in which a scoring system for assessing mammary gland maturity was described (12) and on studies in humans in which digitized mammographic images were evaluated for breast density (13).

Assessment of Mammary Epithelial Proliferation by BrdUrd Labeling. Rats were injected i.p. twice a day for 3 consecutive days with 50 mg/kg of BrdUrd dissolved in saline. After excision, the abdominal-inguinal mammary glands were fixed in methacarn, processed in an autotechnicon, and embedded in paraffin blocks. Tissues were cut into 5-μm sections and placed onto 3-aminopropyl-triethoxysilane-prepared slides. The sections were then heat immobilized, deparaffinized in xylene, rehydrated in descending grades of ethanol, and rinsed in deionized water. The proportion of labeled cells was detected by immunohistochemical staining as described by Eldridge *et al.* (14). Endogenous peroxidase activity was quenched by incubating in 3% H_2O_2. Mouse anti-BrdUrd (Becton Dickinson) was then applied at a dilution of 1:40 for 60 min; this was followed by treatment with biotinylated rabbit antimouse antibody (DAKO Corp.) and streptavidin horseradish peroxidase. The slides were counterstained with hematoxylin, rinsed, dehydrated, and mounted with Permount. Cells that incorporated BrdUrd were identified by brown granules over the nuclei. One thousand nuclei in each of different mammary compartments (*i.e.*, duct, terminal end bud, and lobuloalveolar bud) were counted, and the number that stained positive was noted.

Mammary Tumor Induction and Determination of Proliferative Activity and Apoptosis in Mammary Tumors. Mammary tumors were induced by the intragastric intubation of 10 mg of DMBA at 50 days of age. Animals were palpated weekly, and the time of appearance and location of tumors in the mammary gland were recorded. The experiment was terminated 21 weeks after DMBA treatment. By that time, the development of palpable tumors had plateaued for several weeks across all groups. Only histologically confirmed adenocarcinomas are reported in "Results." Tumor incidences at the final time point were compared by χ^2 analysis, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis, as described previously (15).

Proliferative activity in the mammary tumors was determined by BrdUrd labeling, as described in an earlier section. Apoptosis in paraffin-embedded tumor sections was evaluated following the procedure supplied with the ApopTag 228 kit, which was purchased from Oncor (Gaithersburg, MD). In brief, the principle of the assay is based on the addition of digoxigenin-labeled nucleotides to the 3′-hydroxyl ends of double- or single-stranded DNA, catalyzed by the terminal deoxynucleotidyl transferase enzyme. An antidigoxigenin antibody conjugated to peroxidase and a chromogenic substrate (diaminobenzidine) are then used to detect incorporated digoxigenin nucleotides in cells.

RESULTS

Effect of CLA Feeding on Lipid Content of the Mammary Gland. Recent studies indicated that mice and chickens fed a 0.5% CLA diet showed a 50% reduction in body fat (16). Because deposition of mammary fat is known to regulate epithelial growth in this tissue, we decided to first examine the effect of CLA on the concentration of fat in the mammary gland. Table 1 summarizes the lipid content in the mammary fat pad of rats fed either the control or 1% CLA diet. Both diets were given starting at weaning and continuing for 1 month before the animals were sacrificed. There was no difference in the amounts of total lipid, neutral lipid, and phospholipid that were extractable from the mammary tissue between the two groups. Neutral lipid represented a predominant constituent of total lipid. This is consistent with the fact that triglyceride-containing adipocytes are a major component of the mammary tissue. On the other hand, phospholipid is present at a concentration that is about 100 × less. Epithelial cells are the primary source of phospholipid in the mammary gland. The low phospholipid level is reflective of the incomplete differentiation state of the mammary epithelium seen in a nonpregnant and nonlactating animal. The method used for separating the neutral lipid and phospholipid fractions recovered approximately 90% of total lipid. It is possible that more polar lipids might be left on the column. Our immediate plan is to investigate this in greater detail and to

Table 1 *Lipid content in mammary fat pad of rats fed either control or 1% CLA diet*[a]

Diet[b]	Total lipid	Neutral lipid (mg/g of tissue wet wt)	Phospholipid
Control	266 ± 25	230 ± 20	2.2 ± 0.3
1% CLA	260 ± 16	227 ± 24	2.1 ± 0.3

[a] Values are expressed as mean ± SE (n = 6).
[b] Diets were fed from weaning to 50 days of age.

Table 2 *Conjugated diene polyunsaturated fatty acids (CD-PUFAs) in mammary tissue of rats fed control or 1% CLA diet*[a]

Diet	CD-PUFA[b] (nmol/mg of lipid)		
	CD 18:2	CD 18:3	CD 20:3
Control	2.7 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
1% CLA	176.9 ± 6.0[c]	2.4 ± 0.3[c]	5.6 ± 0.3[c]

[a] Values are expressed as mean ± SE ($n = 6$).
[b] The type of polyunsaturated fatty acid is designated by the length of the carbon chain (18 or 20) and the number of double bonds (2 or 3).
[c] $P < 0.001$.

determine any potential changes in phospholipid composition due to CLA feeding.

Although our previous studies have hinted that the metabolism of CLA may represent a vital step in cancer protection, the characterization of various conjugated diene polyunsaturated fatty acids in the mammary gland of CLA-fed rats has not been delineated. Because the composition of individual saturated and unsaturated fatty acids in the mammary tissue of CLA-supplemented rats under various dietary conditions has been described in our earlier publication (5), the results shown here will focus only on CLA and its metabolites. Banni *et al.* (11) have recently published detailed analytical methodologies regarding the identification of various conjugated diene polyunsaturated fatty acids; the same method was used to quantify these derivatives in the mammary gland of control and CLA-fed rats. As shown in Table 2, in addition to CLA (CD 18:2), two other conjugated diene polyunsaturated fatty acids were detected, an 18-carbon triene fatty acid (CD 18:3) and a 20-carbon triene fatty acid (CD 20:3). All three conjugated diene polyunsaturated fatty acids were present at very low levels in the mammary tissue of control rats. Their concentrations were elevated to different degrees upon supplementation with 1% CLA in the diet: 65-fold with CD 18:2, 6-fold with CD 18:3, and 14-fold with CD 20:3. The above finding suggests that CLA could be desaturated further (from a diene to a triene) and elongated (from an 18-carbon to a 20-carbon fatty acid) while still maintaining the conjugated diene structure.

Effect of CLA on Morphology and Density of the Mammary Epithelium. It is possible that CLA may reduce mammary cancer risk by directly or indirectly inhibiting the expansion and proliferation of mammary epithelial cells. If this were the case, one might expect to observe a less complex mammary ductal-lobular tree and a reduction in proliferative index associated with the growing epithelium (terminal end buds and/or alveolar buds as opposed to the more stable subtending ducts). Fig. 1 shows representative mammary gland whole mounts from control rats or rats fed 1% CLA. A total of 10 mammary glands were examined from each group. Overall, we could not detect any significant differences in the area of the mammary fat pad occupied by the mammary epithelium as a result of dietary treatment. The density of the mammary epithelium was evaluated by densitometric analysis of the digitized images as described in "Materials and Methods." The data in Table 3 are expressed as absorbance per mm^2, giving the mean ± SE as well as the 95% confidence interval of the two groups. Our analysis indicated that there was a 21% reduction in the density of the mammary epithelium in the CLA-treated rats. This decrease was statistically significant ($P = 0.009$).

Table 4 reports the effect of CLA feeding on proliferative activity of the mammary epithelium as measured by BrdUrd labeling. No change was detected in the cells lining the ducts. However, DNA synthesis in the terminal end buds and lobuloalveolar buds was inhibited by about 30% ($P < 0.05$) as a result of CLA supplementation. The multiple BrdUrd dosing method over a 3-day period (see "Materials and Methods") increases the sensitivity of the technique by allowing more cells to be labeled and also provides the advantage of being able to mirror steady-state rather than snapshot information. Additionally, this method evens out the slight variations in proliferative activity of the mammary epithelial cells due to estrous cycle hormonal surges.

Effect of Timing of CLA Supplementation on DMBA-induced Mammary Carcinogenesis. What is the implication of the above morphological and biochemical changes in relation to mammary cancer risk reduction? This question was addressed by the carcinogenesis bioassay. Our primary objective was to determine whether continuous feeding with CLA for 6 months would be more effective in cancer prevention when compared to the 1-month CLA feeding protocol administered during the period of mammary gland morphogenesis. The results of this experiment are shown in Table 5. As indicated in "Materials and Methods," three different schedules of CLA supplementation were instituted: from weaning to 50 days of age (group B), from 55 days of age to the end of the experiment (group C), and from weaning to the end of the experiment (group D). In all three CLA-supplemented groups as well as in the control group, DMBA was administered to the animals at 50 days of age. At this point, it is important to clarify that our previous work has demonstrated that CLA intake did not affect DMBA binding to mammary DNA (3). Additionally, as will be discussed below, CLA has no effect on phase I and phase II enzymes that are involved in the metabolism of DMBA.

Regardless of whether CLA was given according to the protocol of group B or group C, it reduced the total number of tumors by about 50% ($P < 0.05$). These observations are consistent with that described in our previous reports (5, 7). Interestingly, when CLA was started at weaning and continued to the end of the experiment (Group D), this schedule of supplementation produced essentially a magnitude of tumor inhibition (57%) comparable to that seen in groups B or C. In terms of the timing and length of CLA exposure, group D was representative of the sum of groups B and C. Thus, it might have been predicted that the effects at each stage would have been additive; *i.e.,* the magnitude of tumor inhibition in group D would be in the range of 75% [50% inhibition from feeding CLA prior to DMBA, plus an additional 25% (50% of 50%) inhibition from feeding CLA after DMBA]. The fact that the additive effect was not observed suggests that different mechanisms may be operative depending on whether CLA exposure is coincidental with the period of active mammary gland morphogenesis and development (group B) or occurs during the period of tumor progression after the mature gland is exposed to a carcinogen (group C).

Given the somewhat unexpected result obtained in group D, the above mammary carcinogenesis study was repeated to confirm the reproducibility of the findings. When the data were evaluated at week 15 post-DMBA in the second experiment, the same pattern was found to emerge as that reported in Table 5. Specifically, the magnitude of tumor inhibition was again not statistically different between groups B and D; the total number of palpable tumors was reduced by 45% in group B and 50% in group D. Usually, this type of experiment is maintained for 20 weeks or longer after carcinogen treatment to achieve a plateauing of tumor appearance. In this duplicate experiment, we decided to terminate it at week 15 post-DMBA treatment, so that we could harvest a reasonable number of tumors that would be suitable for the BrdUrd labeling and apoptosis assays. A total of 10 tumors were obtained from group A (control), and 10 tumors were obtained from group D (continuous CLA supplementation). These tumors were chosen based on the criterion that they all showed a fairly uniform growth rate as determined by weekly caliper measurements. Our intention was to avoid the abnormally large tumors (which are frequently necrotic) and the very small tumors (which have a tendency to remain static).

Both the BrdUrd labeling and apoptosis results are reported as a

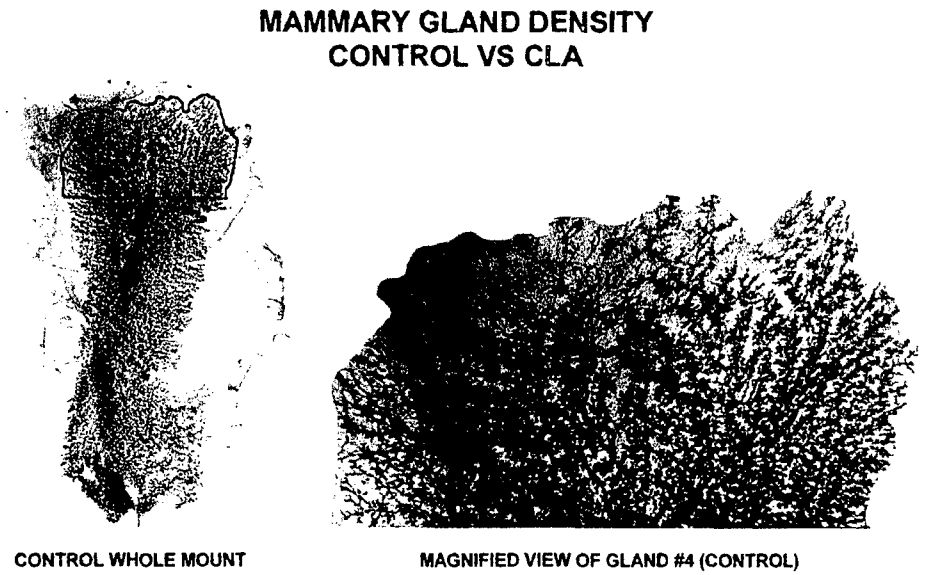

Fig. 1. Representative mammary gland whole mounts from a control rat and a rat fed 1% CLA.

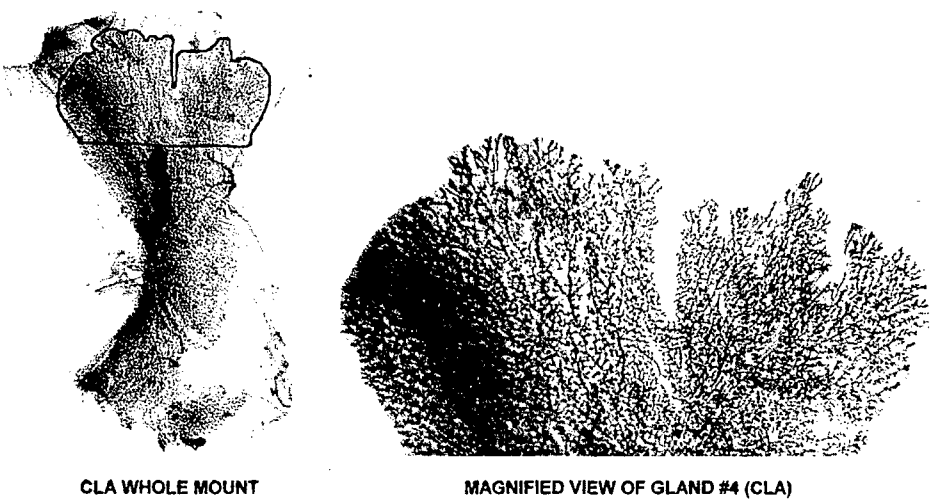

Table 3 *Quantitative analysis of mammary epithelial density in whole mounts of rats fed control or 1% CLA diet*[a]

	Optical density unit per mm^2	
Diet	Mean	95% confidence interval
Control	110 ± 6	96–125
1% CLA	87 ± 4[b]	77–97

[a] The digitized image was taken from photographs as represented in Fig. 1. The data are given as mean ± SE (n = 10) or 95% confidence interval.
[b] P = 0.009 according to Kruskal-Wallis one-way ANOVA.

percentage of cells that reacted positively to the respective immunohistochemical staining methods (Table 6). Although the number of proliferating cells and apoptotic cells appeared to be slightly higher in mammary tumors from CLA-fed rats when compared to those from control rats, the increase was not statistically significant. Whether a larger sample size will add new biological meaning to the above observation remains to be elucidated. Variabilities were generally greater with the apoptosis assay than with the BrdUrd labeling assay. No correlation was evident between the size of the tumor and the intensity of BrdUrd labeling or the extent of apoptosis in either group (data not shown). In general, the data support the hypothesis that tumors growing out in rats fed CLA are resistant to the effect of this fatty acid.

DISCUSSION

Adipocytes are a major and integral cellular component of the rodent mammary gland. They are known to have an important influence on the growth and development of the mammary epithelium. Mammary cells, when transplanted to the gland-free fat pad, will proliferate and expand up to the boundary of the fat pad but not

Table 4 *BrdUrd labeling in mammary epithelium of rats fed control or 1% CLA diet*

	Mammary compartment (% of cells labeled)[b]		
Diet[a]	Duct	Terminal end bud	Lobuloalveolar bud
Control	15.4 ± 1.5	21.7 ± 2.0	26.4 ± 2.1
1% CLA	14.6 ± 1.3	15.5 ± 1.3[c]	18.7 ± 1.9[c]

[a] There were 12 rats used in each group.
[b] A total of 1000 nuclei were counted per mammary compartment.
[c] $P < 0.05$.

Table 5 *DMBA-induced mammary carcinogenesis in rats fed control or 1% CLA diet*

Group[a]	Diet	Duration of CLA feeding	Tumor incidence	Total No. of tumors	% inhibition[b]
A	Control		26 of 30	82	
B	1% CLA	From weaning to 50 days of age	17 of 30[c]	42[c]	49%
C	1% CLA	From 55 days of age to end of experiment	14 of 30[c]	38[c]	54%
D	1% CLA	From weaning to end of experiment	14 of 30[c]	35[c]	57%

[a] DMBA was given to all groups at 50 days of age and the experiment was terminated at 21 weeks post-DMBA.
[b] Percentage inhibition was calculated using the total tumor number data.
[c] $P < 0.05$ compared to the corresponding control group without CLA

beyond (17, 18). Recent studies from Pariza's laboratory indicated that feeding 0.5% CLA in the diet to mice and chickens reduced their body fat by about 50% (16). The above finding prompted us to investigate whether the deposition of mammary fat in rats would be affected similarly. If CLA were to cause a modest reduction of fat deposition in the mammary gland, such an outcome might lead to less than full development of the mammary epithelium, which might explain the decrease in risk to carcinogenesis. This is clearly not the case, as evidenced by the lack of an effect of CLA on the amount of extractable mammary fat (Table 1). We did, however, find a small but significant reduction in the density of the branching mammary epithelium as a result of CLA feeding (Fig. 1; Table 3). The decrease in lateral branching, as best determined by digitized image analysis, should be distinguished from the unaffected ability of the subtending ducts to invade up to the edge of the surrounding fat pad. Thus, our study suggests that CLA has no apparent effect on fat deposition in the mammary tissue but is able to down-regulate the lateral proliferation of the mammary epithelium via either a direct or an indirect mechanism.

The data in Table 4 show that DNA synthesis (determined by BrdUrd labeling) in the terminal end buds and lobuloalveolar buds was inhibited by CLA. It was therefore gratifying to see that quantitative image analysis of reduced morphogenesis in the mammary gland of CLA-fed rats was confirmed by biochemical measurement of a lower rate of cell proliferation. Terminal end bud cells are the progenitors of lobuloalveolar bud cells. In a 50-day-old rat, most of the terminal end bud cells have already been differentiated to lobuloalveolar bud cells (19). Nonetheless, the former structures are the primary target sites for the induction of mammary carcinomas by chemicals. The lower rate of proliferation in the terminal end bud cells could explain in part the decreased risk to chemical initiation of carcinogenesis.

At this point, it is instructive to contrast the differential effects of CLA on DNA synthesis in normal mammary cells (Table 4) *versus* mammary tumor cells (Table 6). One interpretation is that CLA may decrease the turnover of normal cells (*e.g.*, terminal end bud cells and lobuloalveolar cells) and inhibit the clonal expansion of early transformed cells (Table 5, group C) but does not affect the proliferative rate of frank carcinoma cells (Table 6). The last part of the conclusion is logical, because the mere appearance of a tumor in a CLA-treated animal implies that the transformed cells in this tumor have already escaped the suppressive effect of CLA. On the other hand, it should be noted that CLA is able to suppress the growth of a human breast cancer line (MDA-MB-468) transplanted in SCID mice, as reported in a recent study (20). Whether this response is unique to the particular cell line grafted to a severely immunodeficient host remains to be investigated. The low frequency of apoptotic cells in the mammary tumors (Table 6) is not unexpected, because the methodology only provides a freeze-frame picture at a given point in time. Additional research is needed to elucidate whether early transformed cells are more sensitive than frank carcinoma cells to CLA-mediated changes in proliferation and apoptosis.

Table 6 *BrdUrd labeling and apoptosis in mammary tumors from rats fed either control or 1% CLA diet*

		% positive cells[a]	
Diet	Duration of CLA feeding	BrdUrd label	Apoptosis
Control		14.2 ± 1.8	0.6 ± 0.2
1% CLA	From weaning to end of experiment	19.2 ± 2.2	0.9 ± 0.2

[a] Values are expressed as mean ± SE ($n = 10$).

Previous studies have suggested that the metabolism of CLA may be critical in expressing its anticancer activity (21, 22). For example, the conversion of CLA to other related conjugated diene-polyunsaturated fatty acids has a number of potential implications that are relevant to modulation of carcinogenesis. This particular aspect has been discussed in our recent publications (21, 22). Thus, the recovery of desaturation and elongation products of CLA in the mammary gland represents a significant step in this direction. Although CD 18:3 and CD 20:3 were found to be much higher in CLA-fed rats, no CD 20:4 was detected. Suffice it to note that the above analysis was done by using total extractable lipid, which consisted predominantly of neutral lipid because of the contribution from adipocytes. Arachidonic acid is incorporated generally in phospholipid rather than neutral lipid. We are planning to isolate mammary epithelial cells so that a pure phospholipid fraction (free of neutral lipid) can be generated for the analysis of CD 20:4.

Future studies will also focus on the distribution of these conjugated diene polyunsaturated fatty acids in different tissues and blood, as well as in mammary tumors.

The precarcinogen protective effect of CLA (see group B in Table 5) was described initially in rats treated with MNU (7). By using DMBA in the present experiment, we were able to confirm the universality of this response, suggesting that the reduced risk conferred to the animals at an early age is not dependent on carcinogen-specific oncomutations. It is unlikely that CLA achieves this effect by modulating the metabolism of DMBA. As reported previously, CLA does not affect DMBA binding to mammary DNA (3). This observation is consistent with the lack of an effect of CLA on phase I P450 enzymes (1A1, 1A2, 2B1, 2E1, and 3A4),[4] and phase II detoxifying enzymes (23). Of particular interest is the finding that short-term supplementation with CLA prior to DMBA (Table 5, group B) is almost as efficacious as the continuous supplementation protocol (Table 5, group D). One possible reason for this is that exposure to CLA during the time of mammary gland maturation might modify the development of a subset of target cells, such that only CLA-resistant mammary epithelial cells were "available" for carcinogen targeting. This hypothesis could account for the reduced cancer risk of group B and the absence of additional protection seen in group D when CLA was continued after carcinogen treatment. In contrast, both the CLA-sensitive and -insensitive subsets of target cells were present in group C at the time of carcinogen administration. Consequently, the feeding of CLA after carcinogen administration was able to suppress the clonal expansion of those transformed cells that originated from the CLA-sensitive progenitors. This interpretation is supported by the

[4] C. Ip, unpublished data.

data in Table 6, which demonstrate that the proliferative rate of tumors from CLA-fed rats was not inhibited when compared to tumors from control rats. Although somewhat simplistic, the above concept offers a unifying hypothesis to interpret the collective results described in this paper, and is a reasonable starting point to investigate in depth the multiple mechanisms involved in the action of CLA in cancer prevention.

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Decrease in linoleic acid metabolites as a potential mechanism in cancer risk reduction by conjugated linoleic acid

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Previous research suggested that conjugated linoleic acid (CLA) feeding during the period of pubescent mammary gland development in the rat resulted in diminished mammary epithelial branching which might account for the reduction in mammary cancer risk. Terminal end buds (TEB) are the primary sites for the chemical induction of mammary carcinomas in rodents. One of the objectives of the present study was to investigate the modulation of TEB density by increasing levels of dietary CLA and to determine how this might affect the risk of methylnitrosourea-induced mammary carcinogenesis. The data show a graded and parallel reduction in TEB density and mammary tumor yield produced by 0.5 and 1% CLA. No further decrease in either parameter was observed when CLA in the diet was raised to 1.5 or 2%. Thus, optimal CLA nutrition during pubescence could conceivably control the population of cancer-sensitive target sites in the mammary gland. Since both CLA and linoleic acid are likely to share the same enzyme system for chain desaturation and elongation, it is possible that increased CLA intake may interfere with the further metabolism of linoleic acid. Fatty acid analysis of total lipid showed that CLA and CLA metabolites continued to accumulate in mammary tissue in a dose-dependent manner over the range 0.5–2% CLA. There was no perturbation in tissue linoleic acid, however, linoleic acid metabolites (including 18:3, 20:3 and 20:4) were consistently depressed by up to 1% CLA. Of particular interest was the significant drop in 20:4 (arachidonic acid), which is the substrate for the cyclooxygenase and lipoxygenase pathways of eicosanoid biosynthesis. Thus the CLA dose–response effect on arachidonic acid suppression corresponded closely with the CLA dose–response effect on cancer protection in the mammary gland. This information is critical in providing new insights regarding the biochemical action of CLA.

Introduction

A recent epidemiological study in Finland showed that habitual consumption of whole milk is associated with a reduced risk of breast cancer (1). This was a 25 year prospective study involving 4697 women with a mean age of 39 at the time of recruitment and who were cancer free initially. Among individuals with the highest tertile of milk intake, there was a 60% decrease in relative risk. The adjustment for potential confounding factors, such as smoking, body mass index, number of childbirths, nutrients, etc., did not alter the results. It is possible that some covariant with milk was not assessed in this study and, furthermore, the design would not permit the identification of the active constituent(s) involved. Nonetheless, a growing body of evidence during the past decade suggests that milk fat may contain a number of components with anticancer activity, including conjugated linoleic acid, sphingomyelin and butyric acid (reviewed in ref. 2).

Conjugated linoleic acid (CLA) is a term used to denote certain positional isomers of linoleic acid (3). Linoleic acid is an 18 carbon unsaturated fatty acid with two double bonds at positions 9 and 12. In contrast, the two double bonds in CLA are at positions 9 and 11 or 10 and 12, thus giving rise to the designation as a conjugated diene. Milk and other dairy products are good sources of CLA (4) because of the unique metabolic capability of rumen bacteria in converting linoleic acid to CLA via an enzymatic isomerase reaction (5,6). Almost all of the biological research with CLA was done using a commercial preparation which contains a mixture of the 9,11 and 10,12 isomers, although CLA in food is present predominantly as the 9,11 isomer. Despite the similarity in structure between linoleic acid and CLA, their impact on mammary cancer development is strikingly different. In contrast to linoleic acid, which is known to stimulate carcinogenesis over a wide concentration range (7,8), feeding of CLA at $\leq 1\%$ in the diet produces a significant protective effect (9). In the last few years, several groups of investigators have reported successful cancer prevention by CLA in a number of animal models, including tumors of the mammary gland (10), forestomach (11), colon (12) and skin (13).

Previous research from the Ip laboratory showed a unique activity of CLA in mammary cancer prevention in the rat. When CLA feeding was limited to the period of pubescent mammary gland development it was able to confer a lasting protection against subsequent induction of mammary tumors (14). An evaluation of a digitized image of the mammary tree in whole mounts showed that there was diminished morphogenesis and epithelial branching as a result of CLA treatment (15). This could in part account for the reduced susceptibility to cancer induction because of a decrease in the target cell population. The pathobiology of chemical carcinogenesis in the rat mammary gland has been well delineated (16). In this model, terminal end buds (TEB) are the primary sites for the induction of mammary carcinomas. Currently there is no quantitative data on the modulation of TEB density by CLA feeding during mammary gland maturation and, more importantly, how variations in this subset of cellular structures might affect mammary cancer risk. One of

Abbreviations: CD, conjugated diene; CLA, conjugated linoleic acid; MNU, methylnitrosourea; TEB, terminal end bud.

the objectives of the present study was to investigate the dose-dependent effect of CLA on such a relationship.

In an earlier publication, Banni *et al.* (17) reported that CLA can be desaturated and elongated *in vivo* while still maintaining the conjugated diene structure. Since both CLA and linoleic acid are likely to share the same enzyme system for chain desaturation and elongation, it is possible that increased CLA intake may interfere with the further metabolism of linoleic acid. A second objective, therefore, was to determine whether the dose–response effect of CLA on a reduction in mammary cancer risk might be correlated with a particular pattern of tissue CLA and/or linoleic acid metabolites. The above knowledge is critical in providing biochemical clues regarding the mechanism of action of CLA.

Materials and methods

Animals and CLA supplementation

Pathogen-free female Sprague–Dawley rats were purchased from Charles River Breeding Laboratories at weaning. Upon arrival, they were fed either the basal AIN-76A diet (9) or the basal diet containing 0.5, 1, 1.5 or 2% CLA (Nu-Chek, Elysian, MN). All animals were kept on these five different diets for 1 month (i.e. from 23 to 55 days of age) to prepare them for three separate experiments: (i) quantitation of TEB density in the mammary gland (n = 6 rats/group); (ii) mammary carcinogenesis bioassay in which rats were injected (i.p.) with 50 mg/kg body wt methylnitrosourea (MNU) (n = 30 rats/group); (iii) analysis of CLA, CLA metabolites, and linoleic acid metabolites in tissues (n = 6 rats/group). In experiments (i) and (iii), rats were killed after 1 month of CLA feeding; mammary gland, liver and blood were then collected from each animal at the time of necropsy. In experiment (ii), all rats were returned to the basal diet without CLA after MNU administration. They were palpated weekly for the detection of mammary tumors (9). The experiment continued for 23 more weeks before termination. By this time, the development of palpable tumors had plateaued for several weeks across all groups.

Preparation of mammary gland whole mounts

The abdominal–inguinal mammary gland chain was excised in one piece and stretched onto a 75×50 mm microscope slide. The whole mount was fixed in buffered formalin, dehydrated using a series of ethanol solutions and cleared with two changes of toluene. After rehydration, the tissue was stained with alum carmine. A detailed procedure for the methodology was reported previously (15). The outer 5 mm margin of the mammary whole mount was examined by light microscopy using the criteria described by Russo and Russo (18). This area represents the location of most of the actively proliferating TEB structures of the mammary gland for a young virgin rat.

Quantification of conjugated and non-conjugated diene polyunsaturated fatty acids

Mammary fat pad, liver and plasma were stored at –80°C until they were ready for analysis. Total lipid was extracted by the method of Folch *et al.* (19). Free fatty acids were obtained by a mild saponification procedure described by Banni *et al.* (20) and collected in *n*-hexane. After solvent evaporation, the residue was redissolved in CH_3CN/0.14% CH_3COOH (v/v) for injection into the HPLC system. Separation of unsaturated fatty acids was carried out with a Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector 1040M (Hewlett Packard, Palo Alto, CA). A C-18 Alltech Adsorbosphere column (5 µm particle size, 250×4.6 mm) was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70:30:0.12 v/v/v) at a flow rate of 1.5 ml/min. Non-conjugated diene unsaturated fatty acids were detected at 200 nm and conjugated diene unsaturated fatty acids at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.3 s and were electronically stored. Second derivative UV spectra of the conjugated diene fatty acids were generated using the Phoenix 3D HP Chemstation software. These spectra were taken to confirm the identification of the HPLC peaks. Details of the methodology regarding the characterization of conjugated diene unsaturated fatty acids in both reference and biological samples have been published by Banni *et al.* (21). The method of using an HPLC diode array detector system to analyze polyunsaturated fatty acids (especially those present at low levels) is much more sensitive than the conventional GC method which gives a profile of both saturated and unsaturated fatty acids.

Statistical analyses

The CLA dose–response effect on tumor incidence and tumor yield was analyzed by logistic regression and polynomial regression, respectively, as described in a previous report (22). INSTAT software (GraphPad Software, San Diego, CA) was used to calculate the mean and standard error (SE) of fatty acid measurements. One way ANOVA was applied to evaluate the differences between CLA group means and that of the control. This program is based on the Bonferroni method which requires a higher threshold for statistical significance. The increased stringency is achieved by dividing the traditional random chance probability of 5% by the number of comparisons in the data set.

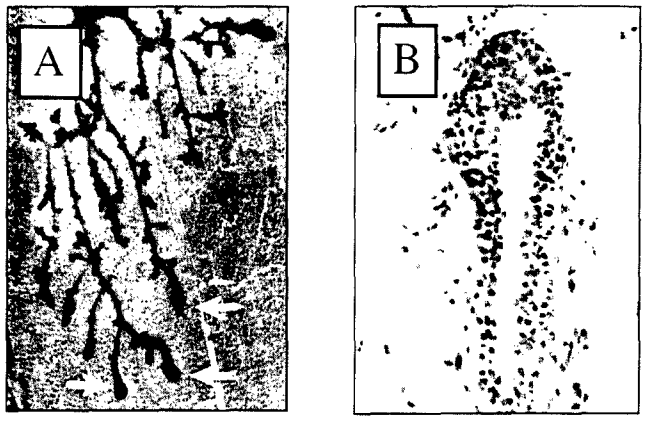

Fig. 1. Terminal end buds in the mammary gland of a 55-day-old rat. (**A**) A number of TEBs (arrows) in a mammary gland whole mount. (**B**) A histological section of a representative TEB under high power magnification.

Results

Reduction in TEB density and mammary cancer risk as a function of CLA intake

Our previous study demonstrated that mammary epithelial branching, as determined by digitized image analysis, was reduced by CLA feeding (15). Since TEBs are the primary sites for the induction of adenocarcinomas in the rodent mammary gland, the first experiment was designed to evaluate the density of TEBs in rats which had been given increasing levels of CLA during the period of pubescent mammary gland development (i.e. from weaning to 55 days of age). Figure 1 shows several TEBs in a mammary whole mount (Figure 1A) and a representative histological section of this structure under high power magnification (Figure 1B). In control rats given a basal diet without CLA, there were 6 TEBs/mm^2 found in the abdominal–inguinal gland, a number that is consistent with that reported by Russo and Russo (18). As can be seen in Table I, increasing the level of dietary CLA to 0.5 and 1% resulted in a graded decrease in TEB density to 5.2 and 4.5 TEBs/mm^2, respectively ($P < 0.05$). No further decrease was observed when dietary CLA was raised to 1.5 or 2%.

What is the implication of this reduction in TEB density in relation to mammary cancer risk? Table I also summarizes the mammary carcinogenesis data in rats which were fed increasing levels of CLA for 1 month from weaning (i.e. the same protocol as in the TEB density study) and then given a single dose of MNU. No CLA was supplied to any of these animals after MNU administration. They were all returned to the control basal diet for the following 23 weeks until the experiment was terminated. There was a progressive inhibition of both tumor incidence and tumor yield as the pre-MNU feeding of CLA increased from 0.5 to 1% ($P < 0.05$; see Table I footnotes d and e). However, no further benefit in cancer prevention was detected above 1% CLA. As shown in Table I, when the amount of CLA was increased from 1 to 1.5 and then to 2% suppression of tumor incidence and total tumor yield appeared

Table I. Reduction in TEB density and mammary cancer risk by CLA as a function of intake[a]

Diet	TEB density[b]	Tumor incidence	Total no. of tumors
Control	6.0 ± 0.2[c]	27/30 (90%)[d]	85[e]
0.5% CLA	5.2 ± 0.2	22/30 (73%)	67
1.0% CLA	4.5 ± 0.2	17/30 (57%)	44
1.5% CLA	4.3 ± 0.1	16/30 (53%)	38
2.0% CLA	4.2 ± 0.2	15/30 (50%)	37

[a]CLA feeding was started from weaning and continued for 1 month (i.e. 23–55 days of age). Mammary whole mounts were prepared from some animals (n = 6/group) for the TEB study. For the mammary carcinogenesis experiment, MNU was injected into each rat at this point (n = 30/group). All animals were returned to the basal diet without CLA after MNU administration and were killed 23 weeks later.
[b]No. of structures/mm^2.
[c]Mean ± SE. Values from 0, 0.5 and 1% CLA groups are different from each other, $P < 0.05$.
[d]Dose-dependent decrease in tumor incidence from 0 to 1% CLA by logistic regression analysis, $P < 0.05$.
[e]Dose-dependent decrease in tumor yield from 0 to 1% CLA by polynomial regression analysis, $P < 0.05$.

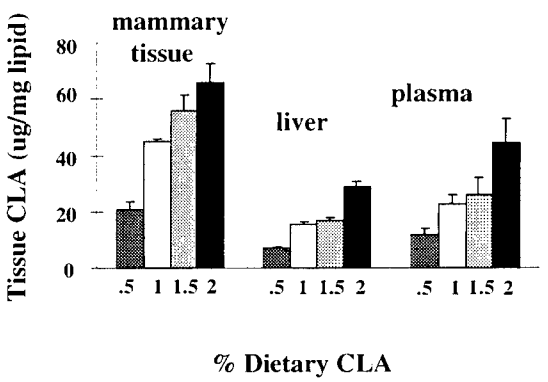

Fig. 2. Tissue CLA concentrations as a function of dietary CLA (mean ± SE, n = 6). In control rats not receiving CLA, tissue CLA concentrations were either undetectable (in mammary gland and plasma) or barely above the detection limit (0.2 μg/mg lipid in liver). These control values are thus not plotted on the chart. The dose-dependent increase in tissue CLA is statistically significant ($P < 0.05$) as determined by regression analysis.

to level off. Thus the anti-carcinogenic response of the mammary gland paralleled closely the reduction in TEB density of the mammary epithelium as a function of CLA intake.

Accumulation of tissue CLA and CLA metabolites as a function of CLA intake

The analytical method for quantifying CLA and CLA metabolites does not distinguish the position of the conjugated dienes (i.e. 9,11 versus 10,12 isomer). Since the commercial CLA preparation used in this study consists of an equal mixture of the 9,11 and 10,12 isomers (9), the analytical data could not provide information on the possible selective incorporation of one CLA isomer over the other or any differences in the conversion of individual isomers to the longer chain metabolites. Thus the results should be interpreted with this limitation in mind even though the terms 'CLA' and 'CLA metabolites' are used here in an inclusive sense to denote specific fatty acids with the conjugated diene structure. In other words, the CLA metabolites reported in the table could have originated from the 9,11-CLA and/or 10,12-CLA, the proportion of which remains unknown with the present method of detection.

Figure 2 shows the progressive elevation of CLA in mammary fat pad, liver and plasma as dietary CLA increased over the range 0.5–2% ($P < 0.05$ in all three tissues). In control rats not receiving CLA, tissue CLA concentrations were either undetectable or barely above the detection limit. For this reason, the control values are not presented in the figure. Compared with the liver, the mammary gland had a higher CLA concentration on a per milligram lipid basis. This is to be expected because we have previously found significant incorporation of CLA into neutral lipids (23), which are the predominant component in mammary tissue. Considering that the mammary gland is essentially a fatty tissue consisting largely of adipocytes, the total amount of CLA stored in the mammary fat pad is substantial. We also examined peritoneal fat in these animals and found that CLA was retained at very similar concentration to that seen in mammary tissue (results not shown). The congruency of the data suggests that adipocyte neutral lipids are a major source of CLA in the body. Finally, the plasma data suggest that CLA levels in the circulation might be a good index of CLA intake.

Figure 3 shows the graded increase in CLA metabolites in mammary tissue and liver as a function of CLA intake. The two metabolites were identified as CD 18:3 and CD 20:3 (the CD prefix indicates the presence of a conjugated diene structure in the fatty acid). They represent the desaturation product (i.e. CD 18:3) and the elongation product (i.e. CD 20:3) of CLA. It is worth pointing out that CLA metabolites were present at a much lower level compared with CLA in both mammary tissue and liver, suggesting that only a small fraction of CLA was metabolized via the desaturation and elongation pathway.

We also analyzed the levels of CLA and CLA metabolites in the abdominal fat pad. The absolute concentrations of CLA, CD 18:3 and CD 20:3 found in this fat depot as a function of increasing CLA intake were very similar to those observed in the mammary tissue (see Figures 2 and 3). This is to be expected because both the mammary gland and abdominal fat pads predominantly consist of neutral lipid-containing adipocytes. Since the abdominal fat pad is not an organ of interest in our research, we choose only to mention its resemblance to the mammary tissue without showing the data.

Changes in linoleic acid metabolites as a function of CLA intake

Figure 4 shows that regardless of the level of intake, CLA did not interfere with the retention of linoleic acid in either mammary tissue or liver, suggesting that CLA was not displacing linoleic acid to any meaningful extent. However, the data imply that the body may handle linoleic acid and CLA differently. The basal diet in this study contained 5% corn oil (AIN-76A formulation). Since ~60% of the fatty acids in corn oil is linoleic acid, the basal diet therefore contained ~3% linoleic acid. As shown in Figure 4, the average linoleic acid concentrations were ~210 and 110 μg/mg lipid in mammary tissue and liver, respectively. In contrast, the data in Figure 2 show that at a level of 2% CLA in the diet, the average CLA concentrations were ~65 and 30 μg/mg lipid in mammary tissue and liver, respectively. Thus a ratio of 1.5 of linoleic acid to CLA in the diet produced a ratio of 3.2 in mammary tissue and a ratio of 3.6 in the liver. The discrepancy between the diet ratio and tissue ratio would suggest that either CLA is not taken up as efficiently as linoleic acid or that CLA is utilized at a faster rate than linoleic acid.

The desaturase and elongase enzyme systems are responsible for the sequential conversion 18:2 (linoleic acid)→18:3→20:3→20:4. Figure 5 shows the changes in

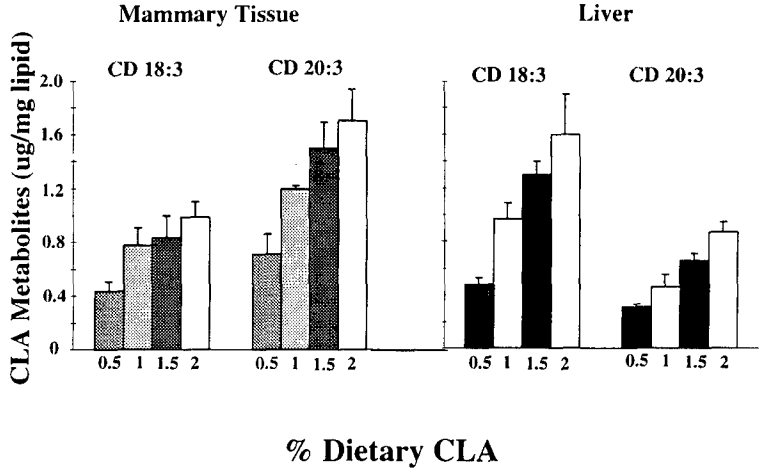

Fig. 3. Tissue CLA metabolite concentrations as a function of dietary CLA (mean ± SE, $n = 6$). CD, conjugated diene. In every case, the dose-dependent increase in tissue CLA metabolites is statistically significant ($P < 0.05$) as determined by regression analysis.

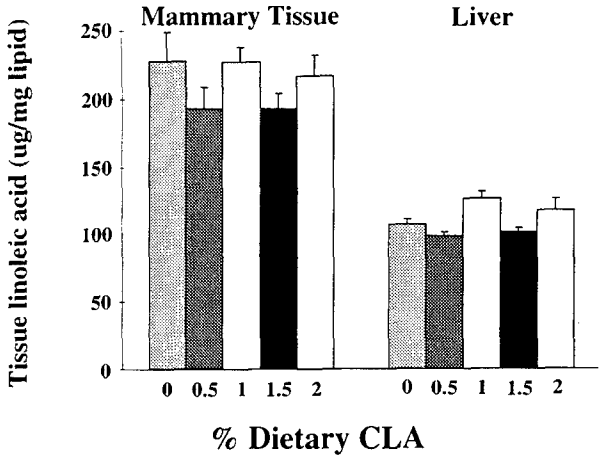

Fig. 4. Tissue linoleic acid concentrations as a function of dietary CLA (mean ± SE, $n = 6$). The differences among groups are not statistically significant from each other.

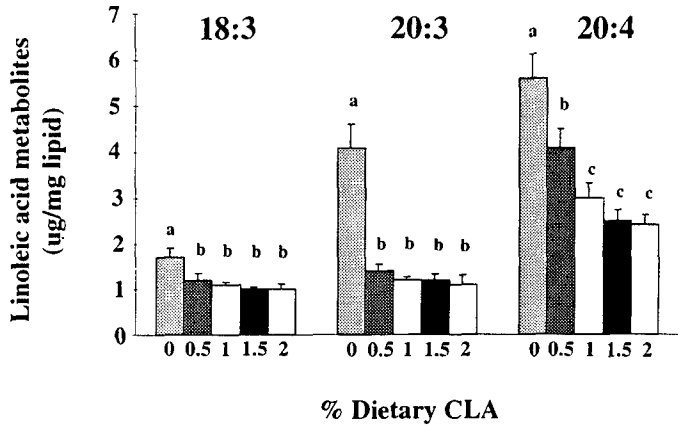

Fig. 5. Changes in linoleic acid metabolites in mammary tissue as a function of dietary CLA (mean ± SE, $n = 6$). Statistically significant differences among groups ($P < 0.05$) are denoted by different letters above each bar.

linoleic acid metabolites in the mammary tissue as a function of CLA intake. In general, the trend indicates a significant drop ($P < 0.05$) in 18:3 and 20:3 up to 0.5% CLA. Of particular interest was the continuing decrease in 20:4 (arachidonic acid) up to 1% CLA. Above 1% CLA there was no further significant reduction in all three metabolites. As expected, the abdominal fat pad behaved very similarly to mammary tissue with respect to the alterations in 18:3, 20:3 and 20:4. However, these changes in linoleic acid metabolites were not seen in the liver (data not shown), suggesting that the effect of CLA on linoleic acid metabolism might be tissue specific, i.e. the effect was greater in neutral lipid-containing tissue (e.g. mammary gland and abdominal fat pad) than in phospholipid-containing tissue (e.g. liver).

Discussion

The results in Table I provide new insights regarding the role of CLA in pubescent mammary gland development and the accompanying modulation of mammary cancer risk. Among the many fatty acids known to influence mammary carcinogenesis (24,25), only CLA has this unique effect in down-regulating mammary epithelial growth during maturation and therefore the size of the target cell population susceptible to carcinogenesis. Computer analysis of a digitized image of a whole mount revealed a 20% reduction in overall branching of the mammary epithelium in rats fed 1% CLA (15). In this study, we were able to obtain quantitative confirmation of a 25% decrease in the density of TEBs. In the developing mammary gland, TEBs differentiate to mature alveolar buds, which in turn give rise to the mammary tree. A lower TEB density is thus consistent with reduced branching of the mammary epithelium. In contrast to alveolar buds, which may become hyperplastic upon transformation by a carcinogen, TEBs are the primary sites for induction of adenocarcinomas. Our data showing a dose-dependent parallel decrease in TEBs and mammary cancer risk produced by CLA support the conclusion that optimal CLA nutrition during pubescence may reduce the number of cancer-sensitive target sites in the mammary gland.

An important issue which remains to be addressed is whether CLA simply slows down the pace of mammary gland development or whether CLA nutrition during pubescence is able to imprint a durable suppressive effect on maturation and proliferative potential of the mammary epithelium. In other words, will the mammary gland in CLA-fed rats eventually catch up and differentiate fully to a stage comparable with that seen in control rats? If so, how will this outcome affect mammary cancer risk? These and other related questions need to be answered because they have far-reaching implications in

terms of pre-teen/teenage CLA nutrition and breast cancer prevention in adulthood.

We have learned in this study that plasma CLA is likely to be a reliable marker of CLA intake. However, the plasma data were obtained at only one time point after 1 month of CLA feeding. Both shorter and longer durations of feeding should be examined in order to evaluate the consistency of the results. We have also found that CLA and CLA metabolites (i.e. CD 18:3 and CD 20:3) accumulate progressively in mammary tissue in proportion to dietary CLA over the entire range 0.5–2%. In contrast, cancer protection by CLA maximized at ~1%, as shown by the data of Table I. Thus the dose–response effect on tissue accumulation of CLA/CLA metabolites might be different to the dose–response effect on cancer inhibition. The significance of CLA/CLA metabolites in perturbing other biochemical pathways in cells at these levels remains to be elucidated.

An interesting point to be brought up here is that we were unable to detect the presence of CD 20:4 (i.e. conjugated arachidonic acid) in tissues of CLA-fed rats. Sebedio *et al.* (26) have recently described the identification of CD 20:4 in the liver of rats given a gavage dose of CLA for 6 days. However, it should be noted that in the above study, rats were kept on a fat-free diet for 2 weeks before and during CLA administration. The absence of competition with linoleic acid (because of the fat-free diet) for the desaturase and elongase enzymes could conceivably facilitate the conversion of CLA to CD 20:4. Polyunsaturated fatty acids of 18 or 20 carbons with a conjugated diene bond are powerful inhibitors of cyclooxygenase and lipoxygenase enzymes (27,28). Thus, the pool of accumulating CLA and CLA metabolites (including CD 18:3 and CD 20:3) may be sufficient to block the biosynthesis of eicosanoids from arachidonic acid via these enzyme pathways.

The ability of CLA to induce a marked decrease in linoleic acid metabolites (i.e. 18:3, 20:3 and 20:4; see Figure 5) in mammary tissue, but not in liver, is a potentially important finding and is worthy of further discussion. Several years ago, Ip *et al.* (29) reported that CLA feeding reduced malondialdehyde, an end product of lipid peroxidation, in mammary tissue, but not in liver. Since only polyunsaturated fatty acids with three or more double bonds are degraded, via peroxidation, to malondialdehyde (30), our present observation of a lower level of total linoleic acid metabolites is consistent with the depressed malondialdehyde levels seen in CLA-treated rats. Thus CLA could attenuate lipid peroxidation in cells by interfering with the formation of linoleic acid-derived polyunsaturated fatty acids which are the substrates for peroxidation. At the present time, we have no knowledge as to whether the decrease in lipid peroxidation contributes to the cancer protective effect of CLA in the mammary gland.

Recently, Belury and Kempa-Steczko (31) described a decrease in arachidonic acid in the liver of SENCAR mice fed CLA. They also found that CLA was incorporated at the expense of linoleic acid. These observations are contrary to our own of a null effect of CLA on linoleic and arachidonic acids in the rat liver. It is possible that SENCAR mice are exquisitely sensitive to CLA-mediated changes in hepatic lipid metabolism. CLA feeding to these mice has been reported to induce hyperlipidemia and the elevated expression of several peroxisome proliferation markers, including acyl-CoA oxidase and fatty acid-binding protein (32). We have no explanation as to why CLA affects linoleic acid metabolism only in the mammary gland in our model. A diminished delivery of linoleic acid metabolites via the circulation appears unlikely because no such changes were detected in the plasma.

The uniform suppression of 18:3, 20:3 and 20:4 by CLA feeding strongly points to a competition between linoleic acid and CLA (as well as their respective metabolites) for the desaturase and elongase enzymes. A study is currently underway using [^{3}H]linoleic acid and [^{3}H]CLA to investigate the kinetics of interaction between these two fatty acids. As can be seen in Figure 5, arachidonic acid levels were decreased only up to 1% dietary CLA. Coincidentally, this sensitivity range corresponded closely with the CLA dose–response effect on cancer protection (see Table I). In both cases, dietary CLA >1% produced little or no further change. It would be reasonable to expect that the biosynthesis of eicosanoids will be affected by the reduced availability of arachidonic acid. In addition to cancer prevention activity, CLA is known to modulate immune functions (33–36), atherogenesis (36) and phorbol ester-mediated events in keratinocytes (37). Eicosanoids are believed to be intimately involved in this spectrum of biological responses. Two recent studies also showed that CLA is capable of reducing the synthesis of prostaglandin E_2 in cell culture (38,39). The data presented here also hint at the possibility that reduced traffic through the eicosanoid pathway may in part be involved in mediating the biological effects of CLA. More in-depth studies are needed not only to assess the modulation of eicosanoids by CLA *in vivo* but also to examine the specificity of different CLA isomers in interfering with this cascade of biochemical reactions.

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Conjugated Linoleic Acid–Enriched Butter Fat Alters Mammary Gland Morphogenesis and Reduces Cancer Risk in Rats[1]

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ABSTRACT Conjugated linoleic acid (CLA) is a potent cancer preventive agent in animal models. To date, all of the in vivo work with CLA has been done with a commercial free fatty acid preparation containing a mixture of *c*9,*t*11-, *t*10,*c*12- and *c*11,*t*13-isomers, although CLA in food is predominantly (80–90%) the *c*9,*t*11-isomer present in triacylglycerols. The objective of this study was to determine whether a high CLA butter fat has biological activities similar to those of the mixture of free fatty acid CLA isomers. The following four different endpoints were evaluated in rat mammary gland: *1*) digitized image analysis of epithelial mass in mammary whole mount; *2*) terminal end bud (TEB) density; *3*) proliferative activity of TEB cells as determined by proliferating cell nuclear antigen immunohistochemistry; and *4*) mammary cancer prevention bioassay in the methylnitrosourea model. It should be noted that TEB cells are the target cells for mammary chemical carcinogenesis. Feeding butter fat CLA to rats during the time of pubescent mammary gland development reduced mammary epithelial mass by 22%, decreased the size of the TEB population by 30%, suppressed the proliferation of TEB cells by 30% and inhibited mammary tumor yield by 53% ($P < 0.05$). Furthermore, all of the above variables responded with the same magnitude of change to both butter fat CLA and the mixture of CLA isomers at the level of CLA (0.8%) present in the diet. Interestingly, there appeared to be some selectivity in the uptake or incorporation of *c*9,*t*11-CLA over *t*10,*c*12-CLA in the tissues of rats given the mixture of CLA isomers. Rats consuming the CLA-enriched butter fat also consistently accumulated more total CLA in the mammary gland and other tissues (four- to sixfold increases) compared with those consuming free fatty acid CLA (threefold increases) at the same dietary level of intake. We hypothesize that the availability of vaccenic acid (*t*11–18:1) in butter fat may serve as the precursor for the endogenous synthesis of CLA via the Δ9-desaturase reaction. Further studies will be conducted to investigate other attributes of this novel dairy product. J. Nutr. 129: 2135–2142, 1999.

KEY WORDS: • *conjugated linoleic acid* • *butter fat* • *mammary gland morphogenesis* • *mammary cancer prevention* • *tissue CLA isomers* • *rats*

Conjugated linoleic acid (CLA)[3] is a term that refers to a collection of positional and geometric isomers of octadecadienoic acid with conjugated double bonds. Milk and other dairy products are good sources of CLA (Parodi 1997) because of the biohydrogenation of dietary unsaturated fatty acids by rumen bacteria. CLA is an intermediate in the biohydrogenation of linoleic acid, and a portion of the CLA in milk fat arises from CLA that has escaped complete rumen biohydrogenation. The other portion of CLA in milk fat is synthesized in the tissues by Δ9-desaturase from *trans*-11 18:1, another intermediate in rumen biohydrogenation (Griinari and Bauman 1999). Recent studies have shown that the milk fat content of CLA can be markedly enhanced by several dietary manipulations, especially those involving dietary additions of plant oils, which are high in unsaturated fatty acids (Griinari and Bauman 1999, Kelly et al. 1998).

CLA is a potent cancer preventive agent (Ip et al. 1994). In animal models of chemical carcinogenesis, CLA has been shown to inhibit skin papillomas (Belury et al. 1996), forestomach neoplasia (Ha et al. 1990), mammary tumors (Ip et al. 1996) and colon aberrant crypt foci (Liew et al. 1995). Moreover, CLA is also effective in reducing the size and metastasis of transplanted human breast cancer cells and prostate cancer cells in SCID mice (Cesano et al. 1998, Visonneau et al. 1997). In the rat model, Ip et al. (1995) also demonstrated that when CLA feeding was limited to only the period of

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[3] Abbreviations used: AUOD, arbitrary unit of optical density; CLA, conjugated linoleic acid; MNU, methylnitrosourea; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome proliferator–activated receptor; TEB, terminal end bud.

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pubescent mammary gland development, it was able to confer a lasting protection against the subsequent induction of mammary tumors. An evaluation of the digitized image of the mammary tree in whole mounts suggested that there was diminished epithelial branching as a result of CLA treatment (Thompson et al. 1997). This change in morphogenesis could account in part for the reduced susceptibility to cancer induction because of a decrease in the population of terminal end buds (Banni et al. 1999), which are the target sites of mammary carcinogenesis.

To date, all of the in vivo work with CLA has been done with a commercial free fatty acid preparation containing a mixture of 8,10-, 9,11-, 10,12- and 11,13-isomers, although CLA in food is predominantly (80–90%) the 9,11-isomer present in triacylglycerols. To date, there has been no information on whether CLA delivered as a constituent of food has biological activities similar to those of the mixture of CLA isomers delivered as free fatty acids. This was one of the objectives; thus, we fed dairy cows in a manner that allowed the production of high CLA butter. In addition to feeding a group of rats the high CLA butter, the design included two other groups that were given either a mixture of CLA isomers (obtained from Nu-Chek-Prep, Elysian, MN, hence designated as Nu-Chek CLA for convenience) or a synthetic CLA preparation consisting predominantly of the 9,11-isomer (obtained from Matreya, Pleasant Gap, PA, designated as Matreya CLA); both preparations provide CLA in the free fatty acid form. A major goal of this investigation therefore was to address the question whether different CLA isomers have different biological activities. The prepubertal rat model alluded to above (Ip et al. 1995, Thompson et al. 1997) was employed for this research. The following endpoints were used to assess the activities of the different sources of CLA: *1*) image analysis of mammary gland development; *2*) terminal end bud (TEB) density; *3*) proliferative activity of TEB cells; and *4*) mammary cancer prevention bioassay. Measurements of CLA isomer incorporation into tissues were also made to provide insight regarding the bioavailability of individual isomers.

MATERIALS AND METHODS

Production and analysis of high CLA butter fat. Holstein cows were used to produce the milk fat that was used to make butter. Cows were located at the Cornell University Teaching and Research Center; this portion of the study was approved by the Cornell University Institutional Animal Care and Use Committee. Control cows (n = 10) were fed a total mixed diet composed of concentrates plus corn silage as the roughage source. Cows (n = 20) used to provide the high CLA butter were fed a similar diet with the addition of 5.3% sunflower oil (Kelly et al. 1998). After 1 wk of consuming the sunflower oil diet, milk samples were obtained and the CLA content of the milk fat was determined. There was substantial individual variation in CLA concentration as reported previously (Kelly et al. 1998). Cows (n = 9) with the highest concentrations of CLA continued to consume the diet for a second week, and their milk was collected to make the high CLA butter.

Raw milk was pasteurized by the high temperature–short time method (model #3919, Alfa-Laval Type-P13-RCF 1982, Kenosha, WI) at 175°F for 18 s, then separated into cream and skim milk. The cream was vat pasteurized at 162°F for 30 min and stored in the cooler for 24 h. It was then churned (Zane Butter Churn Model #A, General Dairy Equipment, Minneapolis, MN) for 30 min at 50°F until butter was the size of popcorn kernels; then the buttermilk was drained off. The butter was rinsed and washed with 4°C water, and the unsalted butter was transferred to 0.5-kg plastic containers and kept at −20°C until use.

Fatty acid methyl esters for butter fat analysis were prepared by the procedure of Christie (1982) and determined by gas chromatography with the use of a Hewlett-Packard GCD system (Palo Alto, CA) equipped with HP G107A GCD software for peak integration (Kelly et al. 1998). Control butter and high CLA butter contained 5.1 and 41.0 mg CLA/g of fat, respectively. The fatty acid composition of these two types of butter is shown in **Table 1**. Saturated fatty acids (4:0–18:0) constituted ~67.7% of the total in control butter fat, but only 48.1% in high CLA butter fat. A major difference was accounted for by palmitic acid, 28.6% vs. 17.8%. The concentration of oleic acid was quite similar, i.e., 18.5% in control butter fat vs. 16.0% in high CLA butter fat. However, there was much more *trans*-octadecenoic acid in the high CLA butter fat (25.0%) than in the control butter fat (3.8%); a major *trans*-isomer was vaccenic acid (*t*11-C18:1). Despite the differences in their fatty acid composition, we decided to use the two kinds of butter fat as is, without making any further adjustment to the fatty acid composition of the rat diet. The argument is that the most important fatty acid for modulation of mammary carcinogenesis in the rodent model is linoleic acid (Ip 1987 and Ip 1997), and the level of this fatty acid was equivalent in the two kinds of butter fat.

TABLE 1

Fatty acid composition of control butter fat and high conjugated linoleic acid (CLA) butter fat

Fatty acid		Control butter	High CLA butter
		g/100 g total fatty acids	
Butyric	4:0	4.2	5.4
Caproic	6.0	2.5	1.4
Caprylic	8.0	1.5	0.7
Capric	10:0	3.5	1.5
Lauric	12:0	4.0	1.7
Myristic	14:0	12.0	7.4
Myristoleic	14:1	1.2	0.9
Pentadecylic	15:0	1.1	0.6
Palmitic	16:0	28.6	17.8
Palmitoleic	16:1	1.4	1.7
Margaric	17:0	0.5	0.3
Stearic	18:0	9.8	11.3
Oleic	*c*9-18:1	18.5	16.0
trans-Octadecenoic[1]	*trans*-18:1	3.8	25.0
Linoleic	*c*9,*c*12-18:2	2.8	2.9
Conjugated linoleic[2]	CD-18:2	0.5	4.1
γ-Linolenic	18:3	0.4	0.2
Others		3.7	1.1
	Total	100.0	100.0

[1] This represents total trans-18:1, of which vaccenic acid (*t*11-18:1) accounted for ~24.8 and 48.7% in control butter and high CLA butter, respectively.
[2] CD denotes conjugated diene.

Experimental protocol for rat feeding studies. Pathogen-free female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) at weaning. Upon arrival, they were divided equally into four dietary groups: *1*) control butter fat, which provided 0.1% CLA in the diet; *2*) high CLA butter fat, which provided 0.8% CLA in the diet; *3*) Matreya CLA; and *4*) Nu-Chek CLA. Matreya CLA and Nu-Chek CLA, purchased from their namesake vendors located in Pleasant Gap, PA, and Elysian, MN, respectively, were added as free fatty acids to the diet to match the total level of butter CLA present in the second group. **Table 2** summarizes the butter fat content of the different diets, the composition of the remaining ingredients, the total CLA level in each diet and the major CLA isomers in the various sources of CLA. It should be noted that the analysis of CLA isomers in the commercial CLA preparations was done by one of the authors (S.B.), and is not based on information provided by the vendors. All rats were fed these four different diets for 1 mo (i.e., from 23 to 55 d of age) to prepare them for the following

TABLE 2

Supplementation of conjugated linoleic acid (CLA) from different sources in the dietary design

Dietary treatment[1]	Total CLA in diet	Group designation	Major CLA isomers[2]	
	g/100 g			%
20% (g/100 g) control butter fat	0.1	Control	83.0	c9,t11
			6.1	t7,c9
20% high CLA butter fat	0.8	Butter CLA	92.0	c9,t11
			4.8	t7,c9
20% control butter fat + 0.7% Matreya CLA	0.8	Matreya CLA	81.0	c9,t11
			17.5	c9,c11
20% control butter fat + 0.7% Nu-Chek CLA	0.8	Nu-Chek CLA	17.6	c11,t13
			36.5	t10,c12
			25.3	c9,t11
			15.3	t8,c10

[1] The remaining ingredients in the diet consisted of 23.5% casein, 44.8% dextrose, 4.1% AIN-76 mineral mix, 1.2% AIN-76A vitamin mix, 5.9% alphacel, 0.3% methionine and 0.2% choline bitratrate.

[2] The butter fat also contained trace amounts (<1%) of cis/trans 8,10-, 10,12- and 11,13-CLA isomers. However, the remaining CLA isomers in Matreya CLA and Nu-Chek CLA are mainly c,c- or t,t-isomers.

four sets of experiments: *1*) digitized image analysis of mammary epithelium in whole mounts, $n = 9$; *2*) quantitation of TEB density in the mammary gland and assessment of proliferative activity in TEB cells, $n = 6$; 3) mammary carcinogenesis bioassay, $n = 30$; and 4) measurements of CLA isomers in tissues, $n = 6$.

Preparation of the mammary gland for image analysis. The abdominal-inguinal mammary gland chain was excised in one piece and stretched onto a 75 × 50 mm^2 microscope slide. The whole mount was fixed in methacarn for 12–18 h and rinsed in 70% ethanol. It was then dehydrated using a series of ethanol solutions (70, 95 and 100%) for 1 h each and cleared in xylene for 2 h. The tissue was rehydrated with descending grades of ethanol and immersed in fresh 0.4% alum carmine stain for 3 d. Once staining was completed, the whole mount was dehydrated using ethanol as described above and cleared with one change of xylene for 2 h. Each whole mount was then placed in a 10 × 15 cm^2 heat-sealable pouch and filled with 20 mL of methyl salicylate. Methyl salicylate was chosen as the clearing agent because its refractory index is very close to that of tissue. This resulted in superior photographic resolution with a clean background. The pouch was left overnight; on the next day, it was pressed flat to remove excess methyl salicylate and air.

Digitization of whole mounts and assessment of optical density of mammary epithelium. All whole-mount images were captured by digital photography (Kodak DCS 420, Kodak Digital Science, Rochester, NY) with the light source passing through the sample from underneath the slides. The Kodak DCS 420 is a digital camera that has a spatial resolution of 1.5 × 10^6 pixels (1012 × 1524) per image. The images were downloaded to Adobe Photoshop (Adobe Systems, San Jose, CA) using a Kodak DCS TWAIN driver. The digitized color images were converted to gray scale (256 shades) images and analyzed by the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). An image-filtering technique was introduced to reduce the intensity variations in the background pixels. Measurements of the mammary fat pad area and the mass of mammary epithelium were performed on the digitized image of the entire abdominal-inguinal mammary gland chain. Mammary fat pad area is defined as the area within the perimeter bounded by circumscribing the outermost terminal end buds of the mammary tree. The mass of mammary epithelium was determined based on the calculation of Σ (a defined area containing mammary epithelial elements × optical density of the assigned area). This integrated value is expressed in arbitrary units of optical density (AUOD). Statistical analysis among groups was done by ANOVA with post-hoc comparisons using Tukey's multiple range test (Zhu et al. 1998).

Quantitation of TEB density. The procedure of preparing mammary whole mounts and staining with alum carmine was similar to that described in the above section. The outer 5-mm margin of the mammary whole mount was examined by light microscopy using the criteria of Russo and Russo (1978). This area represents the location of most of the actively proliferating TEB structures of the mammary gland for a young virgin rat. Images from a minimum of eight fields were transferred from the microscope to a Kodak 8650 PS color printer. Hard-copy pictures were printed out with the use of Adobe Photoshop (Adobe Systems, San Jose, CA). The density of TEB in each field was determined by adjusting the micrometer bar to the power of magnification (Banni et al. 1999). Statistical analysis was done by ANOVA with post-hoc comparisons as described above.

Immunohistochemical staining of PCNA in TEB cells. The proliferating cell nuclear antigen (PCNA) is expressed in early G_1 and S phases of the cell cycle and serves as a good marker for proliferating cells. Methacarn-fixed mammary tissues were processed in a Tissue-Tek Vacuum Infiltration Processor (Miles Scientific, Elkhart, IN) and embedded in paraffin blocks. Ribbons of 5-μm thickness were cut and placed on slides that had been treated with 3-aminopropyl-triethoxysilane. The sections were heat immobilized, deparaffinized in xylene, rehydrated in descending grades of ethanol and rinsed in deionized water and then PBS.

Mouse monoclonal PCNA antibody, purchased from Santa Cruz Biotechnology, (Santa Cruz, CA), was used at a dilution of 1:20,000. Tissue sections were exposed to the primary antibody for 1 h at room temperature in a humid chamber. They were then treated with a biotinylated rabbit secondary antibody against mouse immunoglobulin. This was followed by the addition of streptavidin horseradish peroxidase, which binds to biotin. Diaminobenzidine was used as the chromogen to generate a brown precipitate due to its reaction with peroxidase. All slides were counterstained with hematoxylin, rinsed, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Cells expressing the antigen were identified by a brown stain over the nucleus. Because immunohistochemical staining for a protein is not stoichiometric relative to the amount of protein present, differences in staining were analyzed by using a Kruskall-Wallis rank test as described in detail previously (Zhu et al. 1998).

Mammary carcinogenesis bioassay. Rats in the four different dietary groups were given a single dose (50 mg/kg body weight) of methylnitrosourea (MNU) intraperitoneally at 55 d of age for the induction of mammary tumors. Immediately after carcinogen treatment, all rats were switched to a basal 5% corn oil diet (Ip et al. 1995) without CLA. This diet consisted of 5% corn oil, 20% casein, 65% dextrose, 3.5% AIN-76 mineral mix, 1% AIN-76A vitamin mix, 5% alphacel, 0.3% methionine and 0.2% choline bitartrate. Rats were palpated for mammary gland tumors once a week. The experiment was continued for 24 wk before termination. By this time, the development of palpable tumors had reached a plateau for several weeks across all groups. At necropsy, all tumors were excised and fixed for histological evaluation. Only confirmed adenocarcinomas are reported in the results. Tumor incidences at the final time point were compared by χ-squared analysis, and the total tumor yield was compared by frequency distribution analysis (Horvath and Ip 1983).

Analysis of CLA isomers in tissues. Liver, mammary fat pad, peritoneal fat and plasma were stored at −80°C until they were ready for analysis. Total lipid was extracted by the method of Folch et al. (1957). Free fatty acids were obtained by a mild saponification procedure described by Banni et al. (1994). Methyl esters were prepared by the addition of 14% BF_3/CH_3OH at room temperature and immediately extracted into a solvent consisting of *n*-hexane/water (4:3). After centrifugation at 900 × *g* for 10 min to separate the two phases, the hexane phase was saved and the aqueous phase was further extracted by another round of hexane. The two hexane collections were combined, dried and redissolved in 500 μL *n*-hexane.

Although the BF_3 method causes isomerization of CLA, especially at high temperature (Banni and Martin 1998), we verified the lack of isomerization by BF_3 under the present condition on the basis of the equal analysis of free fatty acid *t,t*-CLA isomers measured in a C-18 column and the methylated *t,t*-CLA isomers measured in a silver-ion column (Banni et al. 1994). Separation of CLA isomers was carried out with a Hewlett-Packard 1050 HPLC system (Hewlett-Packard,

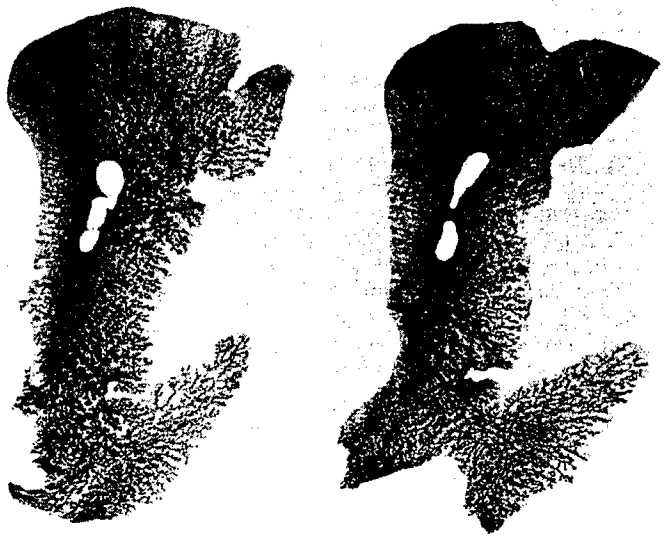

FIGURE 1 Representative mammary gland whole mounts from a control rat or butter conjugated linoleic acid (CLA)-fed rat. The figure shows the entire abdominal-inguinal mammary gland chain from which the lymph nodes had been removed digitally. (Magnification, ×2.3.)

Palo Alto, CA) equipped with a diode array detector 1040M. A silver-ion ChromSpher 5 lipid Chrompack column (Chrompack International BV, Middelburg, The Netherlands), 5-μm particle size, 250 × 4.6 mm, was used with a mobile phase of *n*-hexane with 0.0375% of CH_3CN at a flow rate of 1 mL/min. This technique separates the positional and geometric (*cis* and *trans*) isomers of CLA (Sehat et al. 1998). Conjugated diene unsaturated fatty acids were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were stored electronically. Second-derivative UV spectra of the conjugated diene fatty acids were generated using the Phoenix 3D HP Chemstation (Hewlett-Packard) software. These spectra were taken to confirm the identification of the HPLC peaks. Details of the methodology regarding the characterization of conjugated diene unsaturated fatty acids in both reference and biological samples have been published by Banni and co-workers (1996).

RESULTS

Effect of CLA feeding on mammary epithelial mass and proliferative activity of TEB. CLA feeding had no effect on the growth of the rats. After 1 mo of consuming the different diets starting from weaning, their body weights were: control group, 180 ± 2 g; butter CLA group, 181 ± 2 g; Matreya CLA group, 180 ± 2 g; and Nu-Chek CLA group, 179 ± 2 g. **Figure 1** shows a representative mammary gland whole mount from a rat fed either the control diet or the 0.8% butter CLA diet. Because rats fed a high CLA diet during the 1st mo after weaning developed a less complex network of ductal-alveolar branching regardless of the source of CLA (i.e., butter CLA, Matreya CLA or Nu-Chek CLA), only one example from a high CLA group is presented as an illustration. CLA feeding reduced the development of the mammary tree within the mammary fat pad. The data on epithelial mass of the entire abdominal-inguinal mammary gland chain, as presented in **Table 3**, are expressed in arbitrary unit of optical density (AUOD). There was ~25% lower total mass of mammary epithelium and amount of epithelium per unit area of mammary fat pad in all of the CLA-treated groups compared with the control group ($P < 0.01$). We did not detect any differences in the area of the mammary fat pad among the CLA treatment groups. The results suggest that CLA suppressed mammary branching morphogenesis but did not interfere with the ability of the secondary or subtending ducts to invade up to the boundary of the fat pad.

Our next experiment was designed to determine whether the decrease in mammary epithelial branching was accompanied by a reduction in the density of TEB, which are the primary sites for the chemical induction of mammary adenocarcinomas in the rat model. In control rats, there were an average of 6.3 TEB/mm^2 in the abdominal-inguinal gland at 55 d of age (**Table 4**). CLA feeding resulted in a 30% decrease in TEB density ($P < 0.05$). This magnitude of response was uniform across all of the CLA treatment groups. The proliferative activity of TEB cells was assessed by PCNA immunohistochemistry. Treatment with CLA caused a 25–30% reduction ($P < 0.05$) in the proportion of TEB cells expressing the PCNA antigen.

Modulation of mammary cancer risk by CLA. The mammary carcinogenesis data in rats fed different sources of CLA for 1 mo from weaning and then given a single dose of MNU are summarized in **Table 5**. There was a significant inhibition of both tumor incidence and yield due to pre-MNU feeding of CLA. Overall, CLA treatment decreased mammary cancer risk by ~50%. The different sources of CLA showed similar efficacies as determined by the two variables (tumor incidence

TABLE 3

Quantitative analysis of mammary epithelium density in whole mounts of rats fed different sources of conjugated linoleic acid (CLA)[1]

Group	CLA in diet	Mammary fat pad area	Mass of mammary epithelium[2]	Epithelial mass per unit area of mammary fat pad[3]
	g/100 g	*cm*^{2}	*AUOD*	*AUOD/cm*^{2} × *10*$^{-3}$
Control	0.1	11.8 ± 0.4	1.34 ± 0.09	113 ± 6
Butter CLA	0.8	11.8 ± 0.4	1.04 ± 0.03*	88 ± 1*
Matreya CLA	0.8	11.5 ± 0.4	0.98 ± 0.08*	85 ± 5*
Nu-Chek CLA	0.8	11.5 ± 0.4	0.98 ± 0.06*	85 ± 4*

[1] The digitized image was taken from whole mounts as represented in Figure 1. The data are presented as mean ± SEM, $n = 9$. *$P < 0.01$ compared with the control group.

[2] Mammary epithelial mass was measured in arbitrary unit of optical density (AUOD); refer to Materials and Methods section for details of computation.

[3] AUOD per unit area of mammary fat pad was computed by dividing mass of mammary epithelium by mammary fat pad area.

TABLE 4

Terminal end bud (TEB) density in mammary gland and proliferating cell nuclear antigen (PCNA) expression in TEB of rats fed different sources of conjugated linoleic acid (CLA)

Group	CLA in diet	TEB density[1]	PCNA positive cells in TEB[2]
	g/100 g	*n/mm²*	%
Control	0.1	6.3 ± 0.4	45.2 ± 3.1
Butter CLA	0.8	4.4 ± 0.2*	31.7 ± 2.2*
Matreya CLA	0.8	4.4 ± 0.3*	34.4 ± 2.7*
Nu-Chek CLA	0.8	4.6 ± 0.3*	32.8 ± 2.5*

[1] Results are expressed as means ± SEM, $n = 6$. *$P < 0.05$ compared with control group.
[2] A total of 12–15 TEB structures were counted per rat; each structure consisted of 100–200 cells on a slide.

and total number of tumors) of the bioassay. Thus the data in Tables 3–5 collectively suggest that CLA feeding during pubescent mammary gland development down-regulates mammary epithelial growth, decreases the population and proliferative activity of the target TEB cells, and therefore reduces mammary cancer risk when the rats are challenged with a carcinogen.

Analysis of CLA isomer incorporation into tissues. The total CLA content in tissues of rats fed different sources of CLA for 1 mo from weaning is reported in **Table 6**. As expected, rats fed a high CLA diet had significantly more total CLA in liver, mammary fat, peritoneal fat and plasma. It is noteworthy to point out that rats given butter CLA generally accumulated more CLA in their tissues compared with those given either Matreya CLA or Nu-Chek CLA. The extra load was particularly marked in mammary and peritoneal fat pads. For example, the increase in total CLA content in the mammary fat pad was fourfold by butter CLA, 2.6-fold by Matreya CLA and 2.9-fold by Nu-Chek CLA. In peritoneal fat pad, it was 6.5-fold by butter CLA, 3.8-fold by Matreya CLA and 2.8-fold by Nu-Chek CLA.

The profiles of individual CLA isomers (μg/mg lipid and % total CLA) in the different tissues are reported in **Tables 7–10**. In all three CLA-treated groups, the major CLA isomer found in the tissues was the *c*9,*t*11-isomer. A significant greater percentage of this isomer was found in tissues from the butter CLA and the Matreya CLA groups than in tissues from the Nu-Chek CLA group. The pattern essentially reflected the proportion of the *c*9,*t*11-CLA in the various CLA sources, i.e., 92% in butter CLA, 81% in Matreya CLA and 25.3% in Nu-Chek CLA (see Table 2). The *t*,*t*-isomers represented only a small fraction. The *c*,*c*-isomers were also very low (or sometimes undetectable) in the butter CLA and Nu-Chek CLA groups, but were considerably higher in the Matreya CLA group. This was most likely due to the relatively abundant supply of *c*,*c*-isomer in the Matreya CLA (~18%, see Table 2).

In general, the tissue CLA isomer data in the butter CLA and Matreya CLA groups were quite similar, with the exception of the *c*,*c*-isomer results noted above. A few interesting

TABLE 5

Bioassay of mammary cancer prevention in rats fed different sources of conjugated linoleic acid (CLA)[1]

Group	CLA in diet	Tumor incidence	Total tumors
	g/100 g		*n*
Control	0.1	28/30 (93%)	92
Butter CLA	0.8	15/30 (50%)*	43*
Matreya CLA	0.8	16/30 (53%)*	46*
Nu-Chek CLA	0.8	17/30 (57%)*	48*

[1] CLA feeding was started from weaning and continued for 1 mo (i.e. 23–55 d of age). Methylnitrosourea (MNU) was injected into each rat for mammary tumor induction at this point. All animals were switched to a 5% corn oil diet without CLA after MNU administration and were killed 24 wk later.
* $P < 0.05$ compared with control group.

TABLE 6

Total conjugated linoleic acid (CLA) content in tissues of rats fed different sources of CLA

Group	Total CLA content[1]			
	Liver	Mammary fat	Peritoneal fat	Plasma
	μg/mg lipid			
Control butter	2.6 ± 0.3^{a}	7.2 ± 0.4^{a}	8.8 ± 0.7^{a}	5.4 ± 1.4^{a}
Butter CLA	15.7 ± 1.1^{c}	36.5 ± 2.3^{c}	65.9 ± 1.5^{d}	23.3 ± 2.8^{c}
Matreya CLA	12.5 ± 1.5^{bc}	26.2 ± 1.6^{b}	42.6 ± 4.0^{c}	18.4 ± 3.1^{c}
Nu-Chek CLA	10.2 ± 1.2^{b}	28.2 ± 1.3^{b}	33.4 ± 2.9^{b}	12.5 ± 1.0^{b}

[1] Results are expressed as means ± SEM, $n = 6$. Values that do not share superscript letters are significantly different ($P < 0.05$).

TABLE 7

Conjugated linoleic acid (CLA) isomer concentration in liver of rats fed different sources of CLA[1]

CLA isomer	Dietary group			
	Control	Butter CLA	Matreya CLA	Nu-Chek CLA
	μg/mg lipid			
t,*t*				
11,13	—	—	—	0.1 (0.6%)
10,12	—	—	—	0.2 (1.3%)
9,11	—	0.3 (1.6%)	0.6 (4.7%)	0.1 (0.9%)
8,10	—	0.1 (0.5%)	0.3 (2.5%)	0.1 (0.7%)
7,9	—	0.1 (0.7%)	—	—
c,*t* or *t*,*c*				
11,13	0.1 (2.8%)	0.4 (2.3%)	0.1 (0.8%)	2.1 (20.8%)
10,12	0.2 (8.5%)	—	0.2 (1.5%)	1.6 (16.4%)
9,11	2.0 (76.2%)	13.3 (85.1%)*	8.4 (67.5%)*	4.4 (43.5%)
8,10	0.1 (3.5%)	0.6 (3.8%)	0.4 (3.5%)	1.4 (14.0%)
7,9	0.2 (9.0%)	0.7 (4.2%)	0.2 (1.8%)	—
c,*c*				
11,13	—	—	—	—
10,12	—	—	0.1 (0.8%)	0.2 (1.8%)
9,11	—	0.3 (1.8%)	2.1 (16.9%)	—
8,10	—	—	—	—
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or <0.1%.
* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

TABLE 8

Conjugated linoleic acid (CLA) isomer concentration in mammary fat pad of rats fed different sources of CLA[1]

CLA isomer	Dietary group: Control	Butter CLA	Matreya CLA	Nu-Chek CLA
		μg/mg lipid		
t,t				
11,13	—	—	—	—
10,12	—	—	—	0.3 (1.0%)
9,11	—	0.4 (1.2%)	1.2 (4.6%)	0.4 (1.4%)
8,10	—	—	0.2 (0.7%)	—
7,9	—	—	—	—
c,t or *t,c*				
11,13	—	—	—	5.1 (18.1%)
10,12	—	—	—	3.9 (13.7%)
9,11	6.7 (93.0%)	34.7 (95.2%)*	22.1 (84.4%)*	15.1 (53.6%)
8,10	—	—	0.4 (1.6%)	3.4 (12.2%)
7,9	0.5 (70.0%)	1.3 (3.6%)	—	—
c,c				
11,13	—	—	—	—
10,12	—	—	—	—
9,11	—	—	2.1 (8.2%)	—
8,10	—	—	0.1 (0.5%)	—
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or <0.1%.

* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

points with the Nu-Chek CLA results, however, deserve further comment. The bulk of the CLA isomers in tissues from rats fed Nu-Chek CLA was found as the mixed geometric isomers (i.e., *c,t* or *t,c*), and these will be the focus of this discussion. The percentage of the 11,13-isomer in tissues (20.8% in liver, 18.1% in mammary fat, 19.8% in peritoneal fat and 15.2% in plasma) closely resembled the percentage of the 11,13-isomer present in Nu-Chek CLA (17.6%). Similarly, the tissue incorporation of the 8,10-isomer reflected its proportion in the reagent, i.e., 14% in liver, 12.2% in mammary fat, 14.9% in peritoneal fat and 11.5% in plasma, compared with 15.3% in Nu-Chek CLA. In contrast, the percentage of the 10,12-isomer in tissues (16.4% in liver, 13.7% in mammary fat,16.1% in peritoneal fat and 16.0% in plasma) was much lower than the percentage of the 10,12-isomer present in Nu-Chek CLA (36.5%). It appears that the decrease in the 10,12-isomer was compensated for by an increase in the 9,11-isomer. Thus the percentage of the 9,11-isomer in tissues (43.5% in liver; 53.6% in mammary fat; 43.1% in peritoneal fat; and 49.1% in plasma) was generally much higher than the percentage of the 9,11-isomer present in Nu-Chek CLA (25.3%).

Because the mammary gland is the target tissue of interest, the data in Table 8 will be highlighted in an attempt to gain new insight from the analytical information. The emphasis again will be placed on the *c*9,*t*11-CLA because it is the predominant form. The concentrations of this isomer in the mammary tissue were ~34.7 μg/mg lipid in the butter CLA group and 15.1 μg/mg lipid in the Nu-Chek CLA group. However, the cancer protection benefit was similar in the two groups (Table 5). This is an interesting observation and its implication will be discussed below.

DISCUSSION

This study demonstrates convincingly that milk-fat CLA feeding during the time of pubescent mammary gland development down-regulates morphologic maturation of the mammary epithelium and reduces the risk of mammary cancer in this animal model. To our knowledge, this is the first time that naturally occurring CLA in a food form has been shown to have biological activity. A major attribute of using CLA-enriched milk fat is that the biopotency of CLA can be evaluated in the context of a substance present in our everyday diet. Foods are a relatively inexpensive and effective way in which to deliver substances with cancer protective properties. The concept of CLA-enriched foods could be particularly appealing to people who desire a diet-based approach to cancer prevention without making radical changes in their eating habits. As shown in Table 1, the high CLA butter contains more medium-chain fatty acids than the control butter. We do not believe that this is an important contributing factor to the cancer inhibitory effect of CLA-enriched butter because these fatty acids are not known to modulate carcinogenesis in animal models.

Naturally produced CLA consists predominantly of the 9,11-isomer, whereas the synthetic CLA preparation that is most commonly used in laboratory research contains a mixture of positional isomers. On the basis of a number of endpoints, including mammary morphology, proliferative activity and susceptibility to mammary carcinogenesis, we conclude that the 9,11-CLA isomer is at least as potent as the mixture of isomers in modulating these biological responses at the concentration of dietary CLA provided in this study. There is growing information in the literature on the relationship of

TABLE 9

Conjugated linoleic acid (CLA) isomer concentration in peritoneal fat pad of rats fed different sources of CLA[1]

CLA isomer	Dietary group: Control	Butter CLA	Matreya CLA	Nu-Chek CLA
		μg/mg lipid		
t,t				
11,13	—	0.7 (1.0%)	—	—
10,12	—	—	—	0.4 (1.3%)
9,11	0.1 (1.4%)	0.7 (1.1%)	1.6 (3.8%)	0.4 (1.2%)
8,10	—	—	—	0.2 (0.6%)
7,9	—	—	0.2 (0.5%)	—
c,t or *t,c*				
11,13	—	—	—	6.6 (19.8%)
10,12	0.2 (1.9%)	0.5 (0.7%)	—	5.4 (16.1%)
9,11	7.5 (84.9%)	60.6 (91.9%)*	35.8 (84.0%)*	14.4 (43.1%)
8,10	0.2 (2.0%)	1.3 (2.0%)	0.6 (1.4%)	4.9 (14.9%)
7,9	0.6 (7.2%)	2.2 (3.3%)	—	—
c,c				
11,13	—	—	—	—
10,12	0.1 (1.1%)	—	—	—
9,11	0.1 (1.5%)	—	4.4 (10.3%)	0.4 (1.3%)
8,10	—	—	—	0.2 (0.6%)
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or <0.1%.

* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

TABLE 10

Conjugated linoleic acid (CLA) isomer concentration in plasma of rats fed different sources of CLA[1]

CLA isomer	Dietary group			
	Control	Butter CLA	Matreya CLA	Nu-Chek CLA
	μg/mg lipid			
t,t				
11,13	—	—	—	0.1 (1.1%)
10,12	—	—	—	0.1 (0.9%)
9,11	—	0.6 (2.5%)	0.8 (4.5%)	0.1 (1.1%)
8,10	—	0.2 (0.9%)	—	—
7,9	—	—	—	0.1 (0.9%)
c,t or *t,c*				
11,13	—	0.2 (0.7%)	—	1.9 (15.2%)
10,12	—	0.3 (1.3%)	—	2.0 (16.0%)
9,11	4.5 (82.6%)	20.2 (86.7%)*	14.4 (78.3%)*	6.1 (49.1%)
8,10	—	1.0 (4.4%)	—	1.4 (11.6%)
7,9	0.9 (17.4%)	—	—	—
c,c				
11,13	—	—	—	—
10,12	—	—	—	—
9,11	—	0.6 (2.5%)	3.2 (17.2%)	0.4 (2.7%)
8,10	—	0.2 (1.0%)	—	0.2 (1.3%)
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or <0.1%.

* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

CLA isomer specificity and biological or biochemical effects. At the whole-animal level, the 10,12-CLA isomer has been reported to be responsible for the CLA-induced reduction in body fat accretion in growing mice (Park et al. 1999) and in milk fat synthesis in lactating cows (Baumgard et al. 1999). Park et al. (1999) also showed that in cultured 3T3-L1 adipocytes, *t*10,*c*12-CLA depressed lipoprotein lipase and enhanced glycerol release into the medium. These biochemical responses, however, were not elicited by *c*9,*t*11- or *t*9,*t*11-CLA. On the other hand, recent evidence has indicated that Nu-Chek CLA and Matreya CLA were equally effective in inhibiting growth and inducing apoptosis of rat mammary epithelial cells in primary culture (M. Ip et al. 1999). CLA is a high affinity ligand and activator of peroxisome proliferator–activated receptors (PPAR), a family of transcription factors known to affect gene expression in a tissue-specific manner (Moya-Camarena et al. 1999a). Using a scintillation proximity assay with bacterially expressed human PPARα ligand binding domain, Moya-Camarena and co-workers (1999b) showed a hierarchy of binding affinity for certain CLA isomers in the order of *c*9,*t*11 > *t*10,*c*12 > *t*9,*t*11. In agreement with its high binding affinity, *c*9,*t*11-CLA was also the most efficacious PPARα activator, as determined in a PPARα-reporter gene assay system. This is probably the first study in which the specificity of a CLA isomer is distinguished with the help of molecular tools. Depending on the complexity of the model system, the ability to control for confounding variables and the nature of the assay endpoints, it is not surprising that different conclusions are obtained from the different studies. It does not mean that these conclusions are conflicting. The lesson to be learned is that the data have to be put in the proper perspective so that they can be interpreted appropriately.

On the basis of the analytical data from four different tissues in this study, there appears to be some selectivity in the uptake or incorporation of the *c*9,*t*11-isomer over the *t*10,*c*12-isomer. Studies in lactating dairy cows have also found a similar difference in that the transfer efficiency of a dietary supplement of the 10,12-CLA isomer was only about one half of that observed for the 9,11-CLA isomer (Chouinard et al. 1999). Similarly, in pigs fed a commercial CLA mixture, Kramer et al. (1998) showed that there was a preferential incorporation of the *c*9,*t*11-isomer into liver phospholipids, and of the *c*11,*t*13-isomer into heart lipids. Little information is currently available regarding the biochemical mechanism that regulates the metabolism of the different CLA isomers. Previous research has shown that CLA can be further desaturated and elongated (Banni et al. 1996 and 1999, Sebedio et al. 1997). Some of the ingested CLA is likely oxidized for the production of energy. Future studies should be designed to provide information concerning whether individual CLA isomers are utilized differently via various metabolic pathways.

We have shown that the reduction in mammary cancer risk by CLA under the present experimental conditions is likely due to a decrease in the target cell population, coupled with a lower level of proliferative activity of the target cells. The diminution in TEB density by CLA feeding is consistent with the down-regulation of mammary epithelial branching as determined by digitized image analysis of the whole mount. All of the above variables responded with the same magnitude of change to the different sources of CLA, even though there was more total CLA in the mammary tissue of rats fed butter CLA compared with those fed either Matreya CLA or Nu-Chek CLA. Clearly the 9,11-CLA isomer is biologically active as an anticarcinogen, given the results of the butter CLA. The similarity of the effects of butter CLA and the other two CLA sources suggests that other isomers of CLA may also possess anticarcinogenic activity. However, we cannot rule out the possibility that the 9,11-CLA isomer is already reaching a maximal effect at the tissue level of 9,11-CLA achieved by feeding the Nu-Chek preparation. It would be instructive to carry out dose-response studies with different CLA isomers.

There is one potential advantage to the high CLA milk fat (or butter fat) that merits further attention. Rats consuming butter CLA consistently accumulated more total CLA in their tissues compared with those consuming either Matreya CLA or Nu-Chek CLA. We suspect that this was probably due to the consumption of vaccenic acid (*t*11–18:1) provided by the high CLA butter fat (see Table 1). Vaccenic acid is converted to 9,11-CLA by the Δ9-desaturase enzyme (Griinari and Bauman 1999). The efficiency of this reaction in rodents will be examined in future experiments. Thus, in addition to providing CLA as is, butter fat may also supply the precursor for the endogenous synthesis of CLA. If the rate of endogenous synthesis of CLA is adequate, this pathway may play an important role, which could translate into a potential difference in dose response to CLA-enriched milk (butter) fat vs. synthetically prepared CLA. This represents a new dimension of the project that was not anticipated at its inception. Further studies will be conducted to investigate other attributes of this novel dairy product.

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EFFECT OF CONJUGATED LINOLEIC ACID ON LIPID PEROXIDATION

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Abbreviations: CLA, conjugated linoleic acid; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; FO, fish oil; DP, 2,2'-dipyridyl; BHT, butylated hydroxytoluene; TBA, thiobarbituric acid; AOS, antioxidant solution; BHA, butylated hydroxyanisole; TMP, tetramethoxypropane

Abstract

Two dietary lipids, fish oil (FO) and conjugated linoleic acid (CLA) , that have been shown to inhibit carcinogenesis in experimental animals, have also been reported to have opposite effects on lipid peroxidation. Lipid peroxidation has been hypothesized to be involved in the initiation, promotion, and progression stages of the carcinogenic process. The objective of this in vivo experiment was to determine the effect on lipid peroxidation when FO and CLA were fed alone or in combination. The study was designed to investigate this question under conditions of high oxidative stress. Sprague-Dawley rats were fed one of two basal diets, containing either menhaden oil (FO) or palm oil as the predominant lipid. Each diet was prepared both with and without CLA (1% w/w). Clofibrate was added to the diet (0.125%) to induce oxidative stress. Diets were fed for 4 wk. Liver MDA and 8-OHdG levels were observed to be elevated in rats fed the FO diet; CLA had no effect. The effect of FO and CLA on mammary gland levels of MDA was dramatic. FO increased mammary gland MDA by an order of magnitude ($p<0.01$), and CLA diminished significantly the extend of this increase ($p<0.05$). Thus the effects of FO and CLA on lipid peroxidation were opposing. The most prominent effects on lipid peroxidation were exerted in the mammary gland, a tissue which is rich in neutral lipid. It appears that the antioxidant activity of CLA is primarily limited to neutral lipid. These data provide a rationale for combining FO and CLA in experiments designed to test the effectiveness of combinations of chemopreventive agents against the induction of mammary carcinogenesis.

Introduction

While a considerable amount of attention has been given to the negative impact of dietary lipids on health, certain lipids have been reported to confer protection against a number of disease processes (1-4). Fish oil , and more specifically its omega-3 fatty acid constituents, and conjugated linoleic acid (CLA), a positional and geometric isomer of linoleic acid that is found preferentially in beef and dairy products have both been reported to inhibit carcinogenesis in several experimental models (5-8). While the mechanisms that account for the beneficial effects of these lipids have yet to be identified, one biochemical parameter that both lipids have been reported to affect, albeit in opposite directions, is the peroxidation of lipids (9-12). Lipid peroxidation has been hypothesized to either be a biomarker of oxidative cellular events associated with carcinogenic initiation, promotion and progression, or to be directly involved in carcinogenesis via the induction of genetic mutations by the oxidation of DNA bases or via an epigenetic mechanism(s) (13-16).

A factorial experimental design was used to address several questions relative to the effects of FO and CLA on oxidative cellular events. The questions addressed were: what quantitative differences exist in lipid peroxidation induced by fish oil in a phosphlipid rich (liver) versus a neutral lipid rich (mammary gland) tissue; can the reported antioxidant activity of CLA observed in ex-vivo experiments be detected in vivo; and do changes in the levels of an indicator of DNA oxidation, i.e. levels of 8-OHdG in genomic DNA, parallel changes in lipid peroxidation. In the course of these experiments a previously unreported problem in recovery of the thiobarbituric acid derivative of MDA was discovered; a method to overcome this problem also is reported.

Materials and methods

Animals. Female Sprague Dawley rats were obtained from Taconic Farms (Germantown, NY) . Rats were housed individually in metabolic cages in an environmentally controlled room maintained at 25 C with 50% relative humidity and a 12 hr light-12 hr dark cycle. Distilled water was provided ad libitum.

Diet composition. The dietary formulation that was used is shown in Table 1 and the mixture of oils and CLA that was blended into each diet is shown in Table 2 (17). Menhaden oil and corn oil were protected from oxidation with 0.02% tert-butylhydroquinone (TBHQ). This antioxidant is frequently used in dietary oils , but is minimally retained in animal tissues and is thought to possess little antioxidant activity *in vivo* (18, 19). Diets were stored frozen and were provided fresh every other day. Menhaden oil was obtained from the Fish Oil Test Materials Program of the National Marine Fisheries Service. Corn oil containing no vitamin E was purchased from Eastman Kodak Company, Rochester, NY. Palm oil was obtained from Premier Edible Oils, Portland, OR. CLA was custom ordered from Nu-Chek, Elysian, MN. Clofibrate was purchased from Sigma, St. Louis, MO.

Experimental design: Rats were randomized to experimental groups (n=8-9/group) and adapted to the four experimental diets described in table 2 for 4 weeks prior to the addition of 0.125% clofibrate to the diet which increases hepatic peroxisomal metabolism (20). Following an additional four weeks of feeding the experimental diets, animals were killed, and livers and mammary glands were excised and frozen promptly in liquid nitrogen.

Tissue MDA Determination. Tissue MDA was quantified as its TBA derivative with reverse phase HPLC and photometric absorbance detection at 535nm. Our procedure is based on an extensive modification of the methods described in two publications (21,22). We observed that measurement of tissue MDA was confounded by differences in the protein contents of tissue extracts that were hydrolysed and subsequently derivatized with TBA. By equalizing the protein concentration of the tissue extracts after homogenization, a significant problem of inconsistent MDA-TBA recovery from

tissue extracts was resolved, and a potential source of error eliminated. To our knowledge this issue has not previously been reported in the assessment of tissue MDA.

In detail, mammary gland or liver was homogenized with a Polytron tissue dismembrator in water containing 1% AOS, 1 part tissue to 9 parts water (w/v). For mammary gland, homogenized samples were centrifuged at 6500 x g and fat plugs were removed , followed by further homogenization to re-suspend the pellet. As optimum reaction conditions were found to vary with protein concentration, an amount of homogenate containing approximately 1.2 mg protein was prepared for hydrolysis. The homogenate was combined with 7.5 ul AOS, 7.5 ul 5N HCl, and enough water to bring the volume to 1.5 ml. The covered tubes were heated to 96 C for 3 hours, cooled quickly in tap water, and 30 ul 0.3M Na_2WO_4 per tube was added to facilitate precipitation of protein. After centrifugation at 6500 x g for 10 min 1 ml of supernatant was transferred to a clean glass tube. An aliquot of 0.75 ml TBA solution was added to each tube followed by heating for 90 min at 96E to form the MDA-TBA adduct. Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. Preparation of TMP standards and separation of the MDA-TBA adduct by HPLC was the same as described above. Results are expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (23).

Tissue 8-OHdG Determination. DNA was isolated from tissue with a phenol free process and was subsequently digested enzymatically to nucleosides for chromatographic analysis. The conditions described minimize the ex vivo formation of 8-OHdG. Frozen and pulverized tissue (200 mg mammary gland or 75 mg liver) was mixed with 10ul BHT (26.4 mg/ml) and 3 ml digestion buffer (100mM NaCl; 10mM Tris, pH8.0; 0.5% sodium dodecyl sulfate; 400 ug/ml proteinase K) in a polypropylene screwcap tube. The tube was incubated in a 50E water bath for 16-20 hrs, after which it was removed from the bath and allowed to cool briefly before adding 1 ml 7.5M ammonium acetate and mixing thoroughly. The resulting precipitate was removed from suspension by centrifugation at 19,000 g for ten minutes at 4C, and the supernatant decanted and extracted twice with 24:1 chloroform/isoamyl alcohol. Nucleic acids were precipitated by the addition of 3 ml isopropanol, and the precipitate was washed with 70% ethanol before dissolution in 340 ul TE buffer (10mM Tris; 1mM EDTA; pH 8.0) containing 5mM DP. RNA contamination was reduced by treating samples with RNase (55 ug in H_2O) for 1 hour at room temperature in the dark. After addition of 10 ul of 5M NaCl, DNA was precipitated by the addition of 350 ul isopropanol. While the presence of ribonucleosides does not interfere with the assay per se, removal of most of the RNA by treatment with RNase results in samples which are more readily digested to nucleosides and chromatographed. The DNA pellet was washed with 70% ethanol, dried briefly under reduced pressure without heat, and dissolved in 100ul of 20mM sodium acetate, pH 4.8, containing 5mM DP. Dissolution was allowed to proceed overnight at room temperature in the dark prior to enzymatic digestion to nucleosides, as previously described[15]. 8-OHdG and dG were separated isocratically on a 4.6 X 250 mm Rainin Microsorb C18 column (5um, 100A) with a mobile phase of 8.2% methanol in 50 mM potassium phosphate buffer, pH 5.5, delivered at 1 ml/min. Detection of 8-OHdG was achieved on an ESA Coulochem Model 5100 A electrochemical detector equipped with a model 5011 analytical cell and a model 5020 guard cell. Detector potentials were set as follows: guard cell +0.43 V, detector one +0.12 V, detector two +0.38 V. 8-OHdG was measured as current at detector two. dG was monitored by absorbance at 290 nm with a Shimadzu SPD-10AV spectrophotometric detector installed downstream from the electrochemical detector. Results were reported as number of 8-OHdG per million dG. The simultaneous analysis of both 8-OHdG and dG from a single HPLC injection abrogated the need for a recovery standard or rigorously quantitative sample handling. 8-OHdG was generously provided by R.A. Floyd; dG was from Boeringer Mannheim, Germany.

Data analysis and evaluation. All data were evaluated for distributional normality by probability plot analysis and were determined to be approximately normally distributed. These data were analyzed by factorial analysis of variance and by regression techniques (24).

Results

The effect of FO and CLA on liver MDA and 8-OHdG is shown in Table 3. Liver MDA was approximately 46% higher in rats fed FO versus PO diet ($p<0.001$); a similar effect was not observed on liver 8-OHdG. CLA had no effect on liver MDA or 8-OHdG, although regression analyses were supportive of a positive relationship between liver MDA and liver 8-OHdG ($p=0.031$).

Table 3shows the effect of FO and CLA on mammary gland MDA and 8-OHdG. Rats fed the FO diet had tissue MDA levels that were an order of magnitude higher than rats consuming the PO diet ($p<0.01$). Rats fed the CLA supplemented diet had significantly lower tissue MDA levels ($p<0.05$), an effect that was most pronounced in rats that consumed the FO diet. Within each diet group tissue 8-OHdG tended to be lower in rats fed the CLA supplemented diet ($p=0.08$).

Discussion

Consistent with previous reports, feeding a fish oil formulated diet increased lipid peroxidation measured as MDA in both liver and mammary gland (table 3). However, to our knowledge the magnitude of the difference that FO had on differential MDA in liver, a phospholipid rich tissue, versus MDA in mammary gland, a tissue rich in neutral lipids, has not been previously noted. Whether or not this difference has a bearing on the mechanism of inhibition of carcinogenesis by FO in the mammary gland is unclear, but evidence that lipid peroxides might be involved in the inhibition of the growth of

transplantable mammary tumors has been reported (25,26). The fact that CLA reduced lipid peroxidation in the mammary gland but not the liver also is of interest. While little is known about the biological activity of CLA, this finding is consistent with the results of three previous studies. First, Ip and coworkers have reported that CLA accumulates in tissue neutral lipid rather than phospholipid (27). Thus, the finding that CLA decreased MDA levels in mammary gland but not liver is consistent with its reported tissue distribution. Second, Pariza and coworkers have reported that CLA inhibited oxidation in a test tube antioxidant assay in which lipid peroxidation was the endpoint (28) ; these data were interpreted to indicate that CLA has antioxidant. The work reported here , to our knowledge, provides the first evidence of an antioxidant activity of CLA in vivo. The data reported in tables 3 and 4 also serves to reconcile an apparent contradiction about the antioxidant activity of CLA. Recently it was reported that in an in vitro study designed to model a possible antioxidant role of CLA on membrane phosplipid, CLA was observed to be without effect (29). Specifically, the presence of CLA did not protect membrane vesicles composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine from oxidative modification under various conditions We interpret our data to be consistent with this observation as well as the reported partitioning of CLA among various lipid classes. Thus it does appear the CLA has in vivo antioxidant activity, but that this activity is confined to the neutral lipid component of tissue lipids.

An additional objective of this study was to determine if changes in the levels oxidative damage to DNA measured as 8-OHdG would parallel changes in lipid peroxidation. There are two aspects of the data presented in tables 3 and 4 that merit discussion. First, we used regression analysis to determine if the predicted positive association between tissue MDA and 8-OHdG could be supported statistically (30). The results of those analyses were consistent with the hypothesis of a positive association between these measures of oxidative damage. Specifically those statistical analyses indicated that an animal's tissue concentration of MDA was predictive of the level of 8-OHdG (liver, $p<0.03$; mammary gland, $p<0.0$). Nonetheless, there must be considerable caution in inferring too much about the meaning of this relationship. This point is best illustrated by the data presented in table 4. Levels of mammary gland MDA among groups differed by an order of magnitude whereas levels of 8-OHdG in the same tissues varied by less than 20 percent. One possible explanations for this observation is that 8-OHdG is a DNA adduct and excision repair mechansims exist in the cell such that this adduct can be excised from DNA (31,32). Thus levels of 8-OHdG in genomic DNA are likely to reflect the equilibrium between formation and repair. It was due to this situation that we fed the experimental diets for 4 weeks, so that an equilibrium state would be established. It is also likely that tissue MDA levels represent an equilbirium between formation of the MDA and its further metabolism and elimination (33). Since the pharmacokinetics of both processes are likely to vary, it is not surprising that a straightforward relationship between lipid peroxidation and levels of 8-OHdG was not observed. Thus the finding that an overall association between MDA and 8-OHdG did exist, is consistent with an association between DNA oxidation and lipid peroxidation, but it is clear that different approaches will be needed to determine if this mathematical relationship has a causal basis.

Both CLA and FO have been reported to protect the mammary gland against chemically-induced carcinogenesis and both of these lipids are viewed as chemopreventive agents. One of several approaches in cancer chemoprevention that is being promoted is the use of a combination of agents to increase the probability of inhibiting the carcinogenic response while decreasing the likelihood of adverse effects (33). A desirable element of this approach is the identification of agent combinations based on complimentary mechanisms. The results reported in this paper indicate that a potential adverse effect of fish oil is lipid peroxidation and a potenial benefit of CLA is its reduction of fish oil mediated lipid peroxidation in neutral lipids. A rationale thus exists for evaluating the combined effects of both agents against carcinogenesis, and a logical target for studying the combined effects of these agents is the mammary gland.

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Table 1. Formulation of the diet.

Ingredient[a]	Percent of Diet (w/w)
Solka floc	6.5
Corn starch	18.2
Cerelose	18.2
Casein	26.0
Vitamin mix[b]	1.30
Mineral mix[c]	4.55
DL-alpha tocopherol acetate[d]	0.006
DL-methionine	0.390
Choline bitartrate	0.260
Dietary oil of varied composition (see table II)	24.6

[a] Ingredients are of the grade specified reference 17.

[b]The AIN-76 formulation but with vitamin E omitted.

[C]The AIN-76A formulation as specified in reference 17.

[D]Added only to palm oil containing diets.

Table 2. Dietary Oil Composition

Designated diet	Oil composition (w/w)
PO	21.2 % palm oil, 3.4 % corn oil[a]
PO+CLA	20.2 % palm oil, 3.4 % corn oil, 1.0 % CLA
FO	17.9 % menhaden oil[a], 6.7 % corn oil
FO+CLA	16.9 % menhaden oil, 6.7 % corn oil, 1.0 % CLA

[a] All corn and menhaden oil was free of vitamin E and preserved with 0.02% TBHQ.

Table 3. Effect of diet and CLA on levels of MDA and 8-OHdG in liver.

Diet	CLA	Liver nmol MDA/ mg protein	Liver 8-OHdG/ 10^6dG	Mammary gland nmol MDA/ mg protein	Mammary Gland 8-OHdG/ 10^6dG
PO	No	0.43 ± 0.03	8.4 ± 0.6	0.40 ± 0.03	10.3 ± 0.8
PO	Yes	0.39 ± 0.04	9.0 ± 0.4	0.33 ± 0.02	9.8 ± 0.7
FO	No	0.63 ± 0.03*	9.0 ± 0.4	5.38 ± 0.53	10.1 ± 0.6
FO	Yes	0.61 ± 0.05*	9.1 ± 0.8	1.59 ± 0.23*	8.4 ± 0.8*

Values are mean ± sem of 8-9 animals.

Diet effect on MDA: $p<0.01$.

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Immortalized mouse mammary cells *in vivo* do not exhibit increased telomerase activity

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The acquisition of immortalization is an early and carefully documented event in mouse mammary tumorigenesis. Activation of telomerase activity is one hypothesis to explain the acquisition of immortalization. We examined the activity of telomerase in well-defined immortalized, non-tumor cell populations of mouse mammary tissue *in vivo*. The results indicated that normal virgin and mid-pregnant mammary gland had low to moderate levels of telomerase activity, respectively. In comparison with the levels detected in pregnant mammary gland, telomerase activity was elevated in mammary tumors *in situ* and in established mammary cell lines *in vitro*, both tumorigenic and non-tumorigenic; however, hyperplastic alveolar preneoplastic mammary outgrowths and non-tumorigenic ductal outgrowths, both *in vivo* immortalized populations, had telomerase activity <12% of the levels detected in normal pregnant mammary gland. These results suggest that elevated telomerase activity is not necessary for the immortalization phenotype in *in vivo* mouse mammary cell populations and that elevated telomerase activity occurs as a late event in mammary tumorigenesis. Furthermore, the data suggest that low levels of telomerase activity are characteristic for mouse mammary epithelial cells and not sufficient for immortalization. These data suggest that other factors play more important roles in the induction and/or maintenance of the immortalization state in mammary cell populations.

Introduction

Most cancers are considered to be immortal cell populations. The telomere hypothesis of cellular immortalization is the favorite hypothesis to explain the acquisition of immortalization (1–4). The hypothesis states that telomere size shortens progressively as a consequence of chromosome replication. The end result of telomere shortening is senescence. In order for cancer cells to escape senescence and evolve, the enzyme telomerase is activated and/or increased to stabilize and increase telomere length (2). This hypothesis is supported by the observation that telomerase is activated in tumor virus induced-immortalization of eukaryotic cells (1,4) and that numerous established cell lines and tumors of human origin exhibit increased telomerase activity compared with their normal cells (3). As normal human somatic cells (with some exceptions) exhibit no or extremely low levels of telomerase activity, the results observed in human cell lines and cancer are striking and provide strong support for these hypotheses (3,5,6,7). In the data by Kim *et al.* (3), 98 of 100 immortal human cell populations exhibited positive telomerase activity. This result has been supported and extended by numerous investigators (8–14). In contrast, there are a few reports in the literature where specific neoplasias (15–17), and immortalized cell lines (18) are not associated with increased telomerase activity. Additionally, telomerase activity has been detected in sun-damaged human skin, skin psoriasis and dermatitis, all non-neoplastic conditions (14) and at low levels in hepatitis (12).

The status of elevated telomerase activity of human cancers *in vivo* is consistent among different studies. The status of telomerase activity in pre-malignant human tissues *in vivo* has not been as thoroughly examined. Data in the literature suggest that pre-malignant lesions of pancreas, prostate, colon (adenomatous polyps), stomach, head and neck (erythroplakia) and benign prostatic hyperplasia do not exhibit elevated telomerase activity (8,11,13,19–21). In other experiments pre-malignant lesions of head and neck, designated as either dysplasia or oral leukoplakia, did exhibit elevated telomerase activity in 100% (7/7) and 38% (10/26) of the lesions, respectively (22,23). The mortal or immortalized status of these lesions is unknown. If telomerase function does correlate with immortalization phenotype in human tissues, then one has to conclude that immortalization is a late event in the development of human cancer (2). However, as stated clearly by others (5,24), the data on telomerase activity and immortalization in human tissue is primarily correlative, albeit an extremely strong correlation.

The status of telomerase activity in mouse tumor systems is complicated by the demonstrable levels of telomerase activity in many normal mouse organs (25–27). The considerable background levels of telomerase activity in normal mouse organs requires careful quantification of telomerase in tumor cells. Several reports have demonstrated elevated telomerase activity in viral oncogene and carcinogen-induced mouse skin carcinomas (26–28), carcinogen-induced rat colon tumors (29), viral oncogene-induced pancreatic tumors (28), and mammary tumors from *neu* transgenic mice (26). As in human pre-malignant lesions, elevated telomerase activity was not observed in either pancreatic islet hyperplasia, pre-malignant early stage skin hyperplasia or early stage papillomas, although late stage papillomas did exhibit elevated telomerase activity (26–28). It is worth noting that the immortalized status of mouse skin papillomas is not well studied.

In order to critically evaluate the causal relationship between telomerase and immortalization, well-defined immortalized, non-tumorigenic cell populations *in vivo* are useful. Two recent reports concluded that immortalization of human fibroblasts *in vitro* could occur in the absence of telomerase activation

***Abbreviations:** EL, extended lifespan; TM, transformed mammary; PBS, phosphate buffered saline; PCNA, polymorphic cell nuclear antigen; CHAPS, 3-([cholamidopragh]-dimethylammonies)-1-propane sulfonate; TRAP, telomeric repeat amplification protocol; PCR, polymerase chain reaction; ITAS, internal telomerase assay standard; TBE, Tris-buffer-EDTA; BrdU, bromodeoxyuridine.

but with telomere stabilization (30,31). In these studies, the criteria of immortalization was an *in vitro* criteria and not verified by *in vivo* growth. *In vitro* cell lines are not optimal models because the lack of *in vivo* tumorigenicity of these cell lines is not frequently ascertained and also can be due to culture-induced changes independent of intrinsic tumorigenicity. The mouse mammary system is one of the few models where the acquisition and criteria of immortality in *in vivo* tissues has been studied and carefully documented (32,33). Immortalization has been defined as infinite division potential and is based on orthotopic transplantation of normal or transformed mammary cells into syngeneic mice. The results of such experiments have demonstrated that normal mammary epithelial cells have a finite life span of five to six transplant generations whereas preneoplastic and neoplastic cell poulations can be serially transplanted indefinitely (33,34). Recent experiments have demonstrated that the immortalized phenotype can occur independently of hyperplastic or tumorigenic capability (35). Such *in vivo* cell populations, termed extended lifespan (EL*), resemble normal mammary ducts morphologically and functionally and are absolutely ovarian hormone dependent for growth and morphogenesis. The *p53* status of the EL lines is wild-type (Medina and Butel, unpublished observations).

We have examined the EL ductal outgrowth lines along with established mammary epithelial cell lines *in vitro* and normal, hyperplastic and neoplastic mammary tissue *in vivo* for telomerase activity to test the hypothesis that elevated telomerase activity is necessary and sufficient for immortalization.

Materials and methods

Cells and tissues

All cell lines and tissues were of mammary epithelial origin in Balb/c mice. The biological and tumorigenic properties of the cell lines and tissues have been described previously (35–39). Briefly, the model system examined herein involves mammary tissues that represent different stages in the development of mammary neoplasia. In this model, normal cells give rise to immortalized ductal outgrowths (termed EL), which are ovarian hormone-dependent and weakly tumorigenic and also to immortalized alveolar outgrowths (termed TM for transformed mammary) which are primarily ovarian hormone-independent and tumorigenic. The TM outgrowths represent a heterogeneous group of lesions that have been classified into three stages based on their respective tumorigenic potentials. Stage I lesions are non or weakly tumorigenic, stage II lesions have a moderate (20–70%) tumorigenic potential with a long mean latent period for tumor formation (10–12 months) and stage III lesions have a high (>90%) tumorigenic potential with a short mean tumor latent period (≤5 months). The EL and TM cell populations were derived from normal mature virgin mammary gland and arose 'spontaneously' as a consequence of growth in cell culture. Normal mammary tissues were mature virgin gland (12–16, and 56 weeks of age) and mid-pregnant mammary gland (11–14 days). The immortalized ductal lines EL11 and EL12 were from transplant generations 28–37, preneoplastic hyperplastic outgrowth lines TM2L, TM2H, TM3 and TM40 represented hyperplasias from different stages and were from transplant generations 28–33, 37, 22–27 and 9–15 respectively. Tumors were primary tumors arising from the different hyperplasias.

Cell culture

Virgin mammary gland, EL11 and EL12 ductal tissues were also examined before and after primary culture in three-dimensional collagen gels. The epithelial cells were cultured as described previously (36) and examined at day 0 and day 7 of culture in order to examine the telomerase activity in enriched epithelial cells of normal and immortalized mammary ductal cells.

Proliferation assays

BrdU was purchased from Sigma Chemical Co. (St Louis, MO), and was dissolved in sterile phosphate buffered saline (PBS) at 20 mg/ml. Two-hour pulse-labeling experiments were carried out using 4–6 animals per group. Each animal received 70 μg/g body weight bromodeoxyuridine (BrdU) i.p. The animals were killed, mammary glands were harvested, flattened and fixed in methacarn (methanol:chloroform:acetic acid; 60:30:10) overnight and post-fixed in acetone. Paraffin-imbedded tissue samples were cut in 4 μm thick sections, stained with hematoxylin and eosin for general histological study or used for immunohistochemical staining.

Tissue sections were deparaffinized and blocked as described in (40). BrdU immunohistochemistry was performed using the cell proliferation kit from Amersham following the manufacturer's protocol. The total number of cells counted was 1200 cells per section. For PCNA staining, the procedure was followed as described in (40).

Telomerase assay

Telomerase was assayed by two versions of the telomeric repeat amplification protocol (TRAP) procedure described initially by Kim *et al.* (3) with modification of Wright *et al.* (41). In our initial studies, each sample was washed with 500 μl of ice-cold wash buffer (10 mM HEPES–KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol) and pelleted at 10 000 r.p.m. for 5 min at 4°C. The washing buffer was removed and 300 μl of ice-cold lysis buffer was added (10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenyl-methylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). The samples were homogenized by hand with a glass grinder and placed on ice for 30 min, and then centrifuged at 16 000 *g* for 20 min at 4°C. The supernatants were collected into 1.5 ml tubes and flash-frozen in a dry-ice–ethanol bath. Samples were stored at −80°C. The protein concentration of the cell and tissue extracts were determined with the BCA Protein Assay kit (Pierce) and samples were diluted to a concentration of 1 mg/ml protein. The telomerase assay was performed in two steps; first, a telomerase extension of an oligonucleotide primer (TS) which served as the substrate for telomerase and second, polymerase chain reaction (PCR) amplification with the oligonucleotide primer pair TS 5′-AATCCGTCGAGCAGAGTT-3′ (forward), and CX5′-(CCCTTA)3CCCTAA-3′ (reverse). The PCR buffer contained 20 mM Tris–HCl (pH 8.3); 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20 and 1 mM EGTA. Aliquots of 10 μl of 5XPCR buffer (TRAP buffer) were mixed with 0.1 μg (1 μl) of TS primer, 50 μM each dNTPS (1 μl), bovine serum albumin (0.1 mg/ml), 1 μg of T4g protein (1 μl), 0.4 μl of (α-P32) dCTP (3000 Ci/mmol), 1 μl of a cell extract and 0.5 μl of ITAS DNA (1×10^{-18} g/μl) (added as an internal control). Diethyl pyrocarbonate-treated water was added to bring a total volume of 48.5 μl per assay. The reaction mixture was incubated for 30 min at 22°C for telomerase-mediated extension of the TS primer. Following incubation, 0.1 μg of the CX primer (1 μl) and 2 μl of Taq DNA polymerase (Perkin–Elmer) were added to each tube. The samples were denatured (94°C, 3 min) and subjected to PCR amplification in a thermal cycler for 31 cycles (94°C for 30 s, 50°C for 30 s and 72°C for 45 s). As a positive control, 1 μl of a cell extract from a sample with known telomerase activity (TM-6 mammary tumor cell line) and as a negative control, 1 μl of lysis buffer without cell extract, were used. In addition to the positive and negative samples run in each experiment, experiments assessed the importance of sample size, heat and RNase treatment. Heat and RNase treatment were run on initial experiments using pregnant, virgin and tumor tissue, and, in each case, eliminated telomerase activity. To determine the optimal protein load per sample, for the initial set of assays, pregnant, virgin, EL11 tissue, EL11 and EL12 cultured cells (day 0 and day 7) and TM6 tumor cell line were examined over a range of 0.03 μg to 3 μg. From these experiments, we concluded that 1 μg protein was a reproducible and satisfactory sample size.

In the second set of assays, the same and additional tissue lysates were examined utilizing the TRAPeze™ Telomerase Detection kit (Oncor, Gaithersburg, MD). This kit eliminates the need for a wax barrier hot start and incorporates primers for amplification of a 36-p internal positive standard that provides a positive control for accurate quantitation of telomerase activity with a linear range. Using this kit, we examined a subset of samples over a range of 0.1 μg to 5 μg and picked 2 μg to examine all the samples in two gels run at the same time.

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In the initial experiments, the PCR products were analyzed by electrophoresis of 25 μl aliquots of reaction mixtures on 10% non-denaturing, 0.4 mm thick acrylamide gels, run in 0.5×TBE buffer until the xylene-cyanol had migrated 15 cm from the bottom of the gel. In the second set of experiments utilizing the TRAPeze™ kit, the samples were electrophoresed on 12% polyacrylamide gels, run in 1×TBE buffer until the bromophenal blue marker had migrated 9 cm from the loading wells. The gels were then dried, exposed for 16–20 h to Kodak Biomax film (Eastman Kodak Company). The signal intensity was measured by densitometry. Telomerase activity was determined by adding the intensity of all the bands minus the background observed on the negative control lanes. The signal intensities of the bands from the experimental samples were compared relative to the signal intensity of the bands from the positive controls that were run with each experiment.

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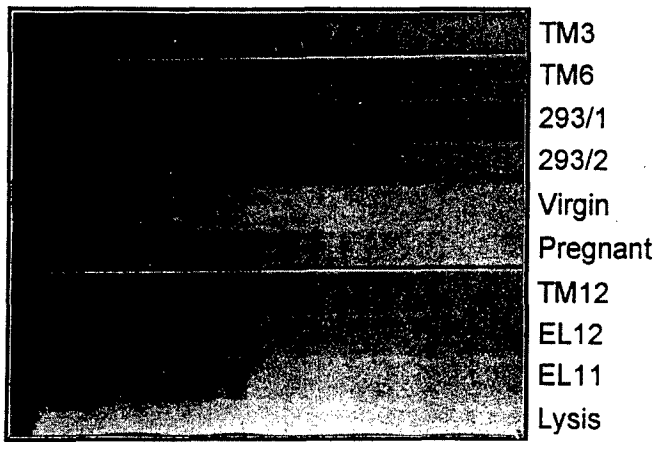

Fig. 1. Telomerase activity in normal (virgin, pregnant), immortal, non-tumorigenic (EL11, EL12) and neoplastic mammary tissues (TM12) and in neoplastic cell lines (TM3, TM6, 293).

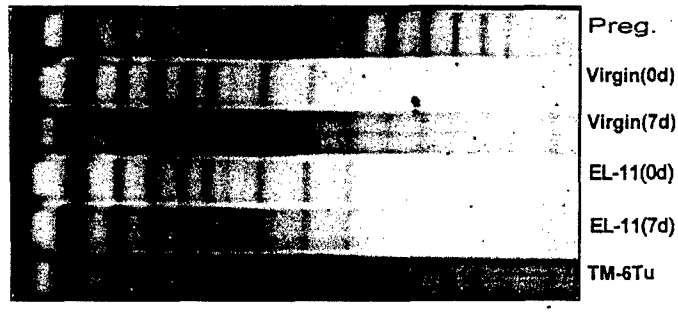

Fig. 2. Telomerase activity in pregnant mammary tissue and in mammary epithelial cells of virgin and EL11 tissues. Day 0 represents the epithelial cell pellet prior to cell culture and day 7 represents after 7 days of culture in a collagen gel.

Results

Telomerase activity in normal and neoplastic mammary tissues and in two cell lines *in vitro* derived from mammary preneoplasias is illustrated in Figure 1. RNase treatment abolished the activity by 99%. Telomerase activity was present in normal pregnant mammary glands, present weakly in virgin gland (18% of pregnant gland), and at high levels in a TM12 primary mammary tumor (1.7 times greater than pregnant) and in the two preneoplastic cell lines (TM3, TM6; 2.1 times greater than pregnant gland). The 293 human kidney carcinoma cell line was an additional positive control. Levels of telomerase activity in mammary fat pads containing EL11 and EL12 ductal outgrowths gave telomerase activities less than pregnant gland (43% less) and slightly higher than virgin mammary gland.

The epithelial cell compartment of the normal mammary ducts as well as the EL11 and EL12 ductal outgrowths in the mammary fat pad contribute in the order of 10–15% of the total protein mass due to the large contribution of the adipose stroma. In order to rule out the possibility that the low level of telomerase activity was an artifact that reflected the low percentage of ductal epithelial cells, the epithelial component of the mammary gland that contained normal ducts, EL11 and EL12 ductal outgrowths were enriched by enzymatic digestion of the gland and only the epithelial cell pellet was collected. The telomerase activity of the pellets was examined at day of collection (day 0) in three experiments and after 7 days of culture (day 7) in a collagen gel in two of the three experiments. The samples at day 7 of culture represented an increase of epithelial cells caused by active proliferation in the collagen gel with growth factors. Figure 2 illustrates the results of one of these experiments. Telomerase activity was evident in the pregnant mammary gland, it was lower in day-0 virgin epithelial cells (26% of pregnant gland) and day-0 EL11 epithelial cells (18% of pregnant gland), and highest in the TM6 tumor cell line (1.25 times greater than pregnant gland). Culturing the virgin and EL11 ductal outgrowths for 7 days increased the telomerase activity 3.2 times in each case. A repeat of the 7-day culture protocol reproduced the same increase in telomerase activity in both cell populations. The mean telomerase activity of the three zero day experiments for both virgin and EL11 cells was 48% of the pregnant gland. The results demonstrated that telomerase activity was not increased in immortalized, non-tumorigenic mammary epithelial cells.

In order to more carefully quantitate the extent of telomerase activity, we utilized the TRAPeze^{TM} Telomerase Detection kit, which contains an internal standard at 36 bp. Figures 3 and 4 illustrate telomerase activity in normal mammary gland, ductal and alveolar hyperplasias and their derivative tumors. The results show that the cleared mammary fat pad, devoid of epithelia parenchyma, has no detectable activity compared with the low activity in virgin and moderate activity in pregnant gland. The activity in virgin gland was 27% of that in the pregnant gland. Of the six separate ductal and 15 separate hyperplastic outgrowth samples, the mean telomerase activity was 12% of the virgin duct and 12% of the pregnant gland, respectively. The two samples of hyperplasias with modest telomerase activities, were detected in two transplant generations of TM2H and TM3H outgrowths that produced palpable tumors within 4 months after transplantation. In contrast, 15 of 16 tumors exhibited elevated telomerase activity with a mean activity 3.7 times greater than the pregnant gland. The specificity of the assay was confirmed by heat inactivation of telomerase enzyme. Figure 5 shows the titration of the telomerase activity over 0.1 to 5 μg of protein and demonstrates that telomerase activity was not artificially missed or exaggerated in any of the samples. A summary of the relative levels of telomerase activity in the mouse mammary tissues is illustrated in Figure 6. The results indicate telomerase activity in the immortalized non-tumor populations is $\leq$12% of that detected in normal, mortal pregnant mammary gland.

The relationship between proliferation status and telomerase activity is illustrated in Table I. The pregnant mammary gland has significant proliferative activity as measured by BrdU labeling and PCNA-labeling indices. It is of note that the percentage of normal cells (pregnant) that are at some stage in the cell cycle, as reflected by PCNA-labeling, is equal to or greater than the hyperplasias but less than primary tumors. The normal virgin duct and immortalized duct show PCNA-labeling indices 6.2 and 24% respectively (42). However, the high BrdU- and PCNA-labeling indices and the low telomerase activities of stage II and III hyperplasias suggest there is little direct correlation between the two cellular processes (Table I).

Discussion

The experiments reported herein examined telomerase activity in a well-characterized model of multistage murine mammary tumorigenesis *in vivo*. This model is characterized by the presence of discrete and well-defined *in vivo* cell populations

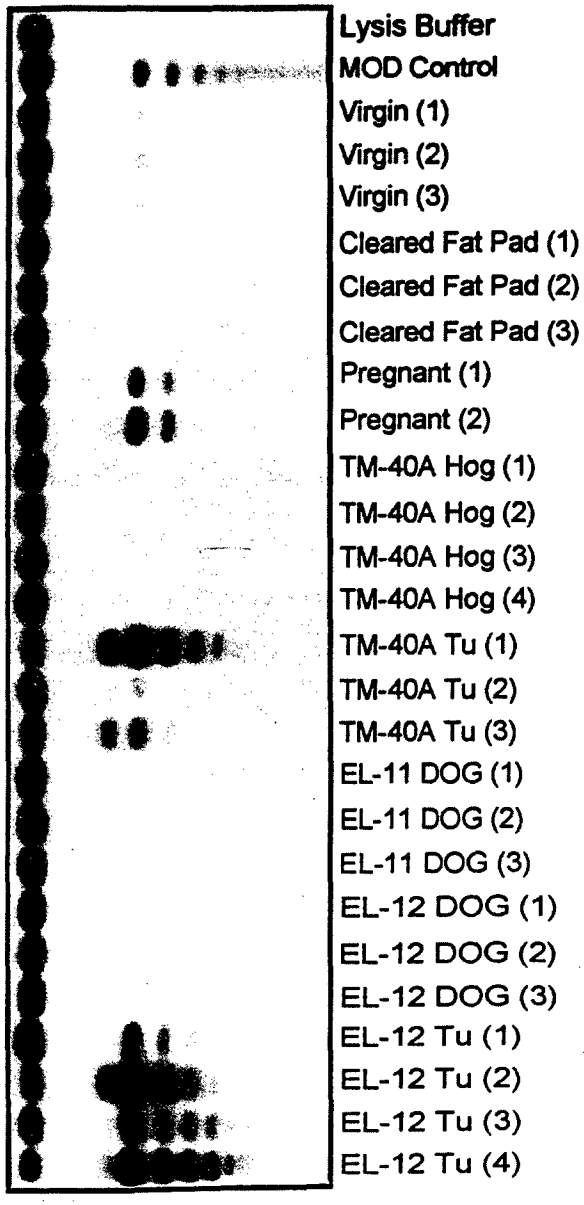

Fig. 3. Telomerase activity in multiple samples of mouse mammary tissues *in situ*. Each lane contains a separate tissue sample. The bottom band in each lane represents the 36 pp internal positive control. HOG refers to alveolar hyperplastic outgrowths, DOG refers to ductal outgrowths and Tu refers to tumor. TM40A is a stage II hyperplasia and EL11 and EL12 are separate immortalized ductal outgrowths. The MOD is a mouse mammary cell line used as a known telomerase positive control.

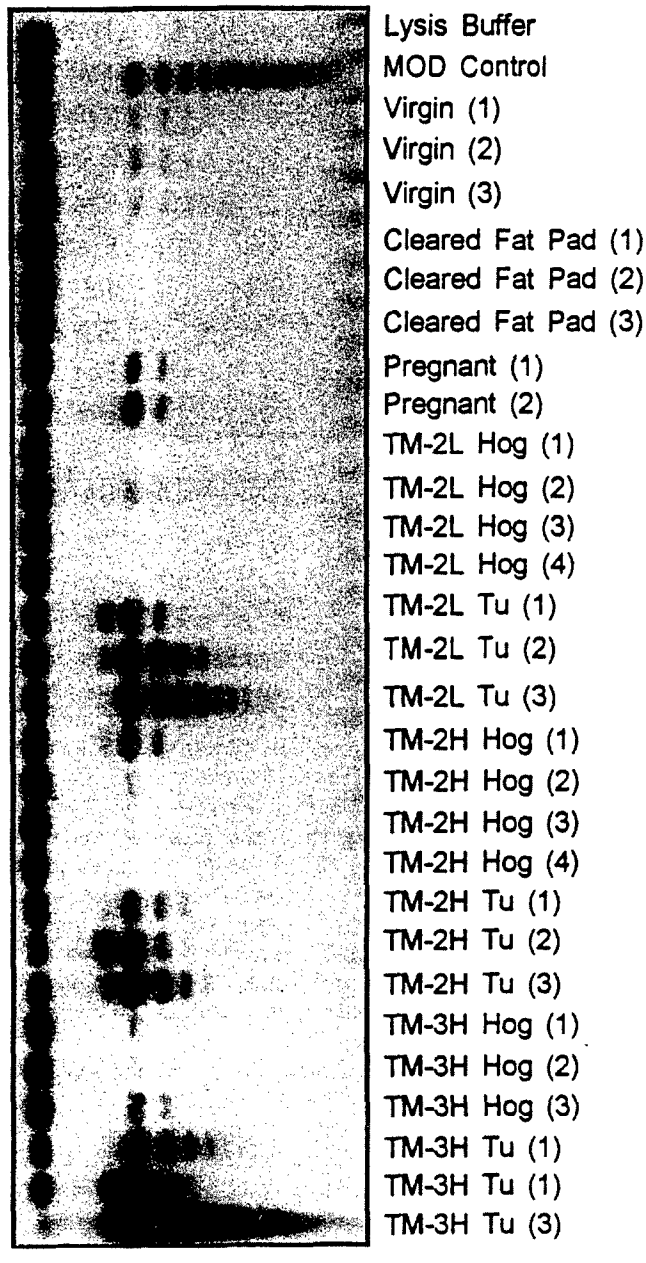

Fig. 4. Telomerase activities in multiple samples of mouse mammary hyperplasias and tumors *in vivo*. The TM2L and TM3H hyperplastic outgrowth lines are stage I and stage II lesions respectively, and the TM2H line is a stage III lesion.

representing four stages in the evolution of normal to neoplastic mammary cells. The experiments specifically addressed the question of whether increased telomerase activity was detectable in immortalized non-tumorigenic mammary ductal cells. The results show that enhanced telomerase activity does not occur in these immortalized cell populations *in vivo*. To our knowledge, this is the first examination of telomerase activity in a known cell population of immortalized, non-tumorigenic cells *in vivo*.

The results raise the question of whether this system is the exception to the rule that telomerase activity is necessary and sufficient for cell immortalization. There is extensive data that immortal tumorigenic cell lines *in vitro* exhibit enhanced telomerase activity (3,5,6). There is also substantial data that immortal, non-tumorigenic cell lines *in vitro* exhibit telomerase activity. However, the relevance of *in vitro* cell lines to growth, immortalization and tumorigenicity can be complicated by selection artifacts found in cultured cells. It is not known whether immortalization *in vitro* measures the same biological phenotype as immortalization *in vivo*. Establishment of a cell line *in vitro* essentially selects for a stem cell population grown under the most elemental conditions; i.e. cells in isolation grown in two dimensions under a minimal set of growth factors. However, immortalization of epithelial cells *in vivo* requires cells to grow in a normal micro-environment in a three-dimensional organization and subject to the influences of neighboring cells and complex hormonal and growth factors. Growth *in vivo* is thus a more stringent test for immortalization and mimics the event that occurs in the evolution of neoplasia.

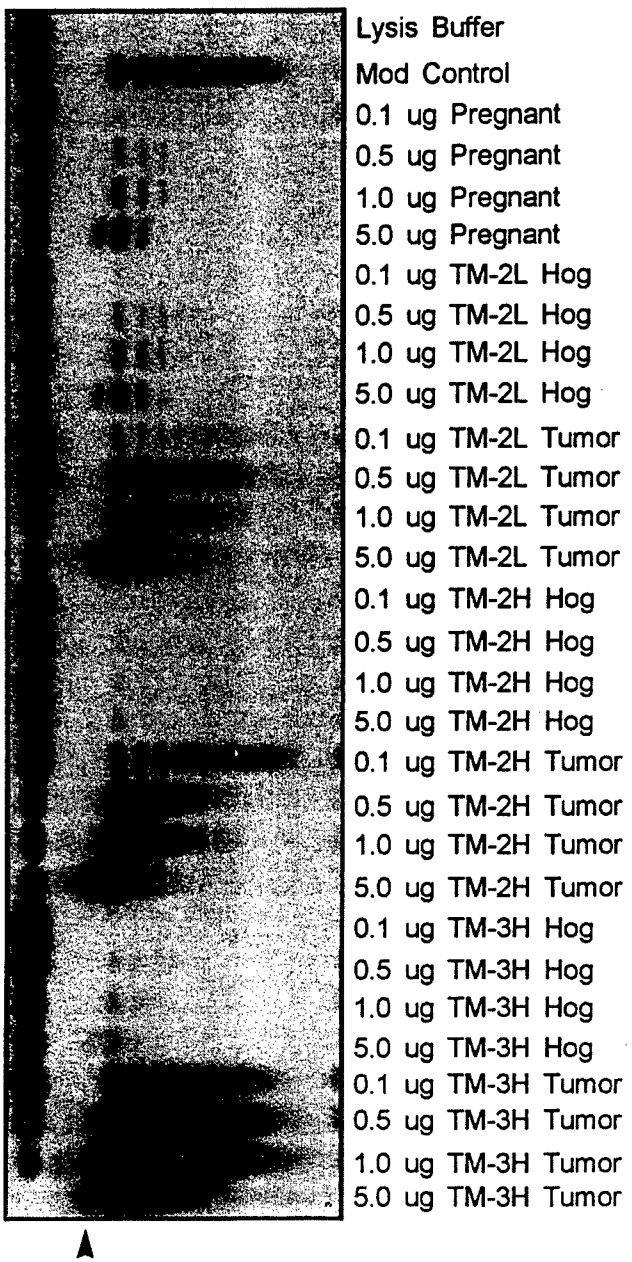

Fig. 5. Representative samples of each mammary stage were titrated for telomerase activity over a range of protein input from 0.1 μg to 5 μg. In some cases, a heavy protein load led to the appearance of a non-specific band (arrow).

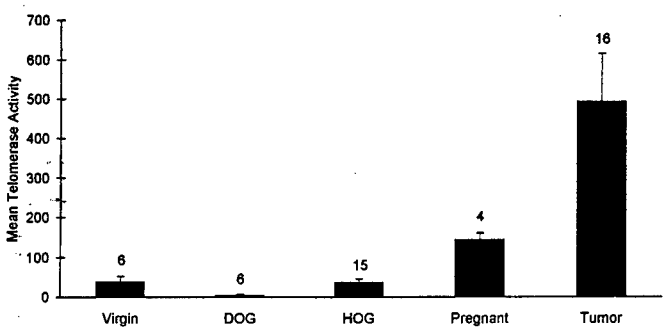

Fig. 6. Summary of telomerase activity in mouse mammary tissues and cell lines. The data are expressed as the mean ± SEM. The numbers above each bar represent the number of individual cell populations sampled for this analysis.

Table I. Proliferation measurements of mammary tissues

Tissue	Proliferation index (%)	
	BrdU	PCNA
Pregnant	9.4 ± 1.4 (3)	46.5 ± 2.6 (4)
TM hyperplasia I, II	7.4 ± 2.4 (14)	24.3 ± 5.6 (13)
TM hyperplasia III	11.4 ± 2.3 (13)	50.3 ± 2.7 (2)
TM tumors	11.4 ± 3.4 (7)	81.3 ± 11.7 (7)

So far, the only system that *in vivo* immortalization has been carefully examined in is the mouse mammary system (32–34,39). The mammary system fulfils the requirements to test vigorously the role of telomerase activation in immortalization and tumorigenesis. The current data suggest that increased telomerase activity is not necessary for the immortalization phenotype in mouse mammary cells as demonstrated by the normal levels of telomerase activity in both immortalized, non-tumorigenic mammary ductal outgrowths and in immortalized, preneoplastic mammary outgrowths.

A second question is whether the low level of telomerase activity present in normal mammary cells is necessary for immortalization. This is a more difficult question to answer by just measuring telomerase activity. Two facts are worth noting. First, the low level of nascent telomerase activity is clearly not sufficient for immortalization as it is well established that virgin and pregnant mammary epithelial cells are mortal cell populations with a life span measured as six transplant generations (32,33). The absence of telomerase activity in the cleared fat pads argues that the telomerase activity measured in the gland is of epithelial, not stromal origin. Secondly, it is curious that the level of telomerase activity in the ductal and alveolar outgrowths was actually less than in their respective normal cell controls. This result argues that even the low level of telomerase activity is not maintained or necessary for an immortalized phenotype. The low levels of telomerase activity may reflect other physiological influences on normal cell function such as hormonal regulation of telomerase activity. Recent results have suggested that varying levels of telomerase activity in normal human endometrium and rat prostate reflect hormonal status of the host (43–45). In addition, telomerase activity can be downregulated in some types of leukemias exposed to differentiation-inducing agents (46,47).

The data reported herein support the emerging data in the literature that telomerase activation in cell population *in vivo* is a late stage event. Thus, the data in mammary cells is consistent with the results in transgenic mouse models of epidermal carcinogenesis and pancreatic carcinogenesis (28), and in carcinogen-induced mouse epidermal carcinogenesis (27). It is also consistent with results published regarding pre-malignant lesions in human cancer (11,19,23). It is important to note that in none of the above organ systems could the immortalized phenotype be rigorously assessed, therefore the relationship between telomerase activation and immortality could not be directly addressed in these systems.

The data are also consistent with reports in the literature that normal mouse mammary tissues exhibit significant telomerase activity (26,48). An apparent discrepancy is the recent report by Broccoli *et al.* (48), which reported little telomerase activity in normal virgin gland and *wnt*-1 hyperplastic mammary glands. The very low levels in normal gland reflect the low

epithelial cell component of the virgin mammary gland and are not necessarily a direct measurement of intrinsic telomerase activity in mammary epithelial cells. The low levels in hyperplastic gland are similar to the results reported with the TM hyperplastic lines. The telomerase activity in the TM hyperplasias is 12% of the activity of tumors; this level is similar to the telomerase activity of *wnt*-1 hyperplasias, which was 14% of telomerase activity in *wnt*-1 mammary tumors (48).

The correlation between telomerase activity and the proliferation status of the cells is ambiguous and complex (3,4,41,49). Some conditions that increase proliferation may also be associated with increase in telomerase activity, but these are distinct from immortalization. Thus, the high levels of telomerase activity in 11 to 14 day pregnant mammary gland may be linked to either the proliferative state of the cells and/or to hormonal stimulation of the gland. Also, the simple process of culturing mammary cells *in vitro* enhances telomerase activity in normal mammary cells as evidenced herein and by Chadeneau *et al.* (26), in immortal mammary cells and established non-tumorigenic cell lines. Alternatively, immortalized cell populations with very low telomerase activities may have proliferation indices that vary from low to high. Thus, proliferation indices of the TM hyperplastic glands are similar to those of the mid-pregnant gland, although they are lower than those observed in tumors. At the other extreme, immortalized ductal outgrowths have very low telomerase activities, with PCNA-labeling indices that are 50% of that found in pregnant gland (38). With respect to tumors, it is likely that the high level of telomerase activity reflects multiple levels of dysregulation of proliferation controls. This possibility is evidenced in the results of Broccoli *et al.* (48) who have shown that the levels of telomerase RNA are slightly elevated (two times) in tumors compared with hyperplasias but telomerase activity is elevated eight times. Similarly, the levels of cell-cycle regulatory proteins and their kinase-associated activities are elevated much higher in these mammary tumors than is suggested by an examination of proliferation indices (40).

It is important to note that the data do not address the question of telomere length as the critical determinant in the acquisition of immortalization. It is conceivable that telomere maintenance in mouse mammary cells is regulated by mechanisms other than the telomerase enzyme. Examples of potential alternative mechanisms have been discussed by Bryan *et al.* (30).

In summary, the results demonstrate that elevated telomerase activity is not obligatory for the establishment of the immortalized state in mouse mammary cells *in vivo*. One has to consider either that the mouse mammary system is an exception to the rule or that telomerase activity reflects altered growth regulation of the tumor cell and is not causal to the acquisition of immortalization. If the latter, attention should be focused on other mechanisms, as emphasized by other investigators (30,50). The role of telomerase in chromosomal segregation in mitosis is one example of a different role for telomerase (50). If the former, then it is necessary to test the telomerase hypothesis of immortalization in carefully defined *in vivo* systems. Regardless of the answer, it is still important to recognize that telomerase activation is a very frequent event in neoplastic cells of human and murine origin and thus provides an appropriate and inviting target for chemotherapeutic or chemopreventive intervention.

Acknowledgements

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MOLECULAR CARCINOGENESIS 20:204–215 (1997)

Gene Expression Changes Associated With Chemically Induced Rat Mammary Carcinogenesis

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Experimentally induced models of breast carcinogenesis in the rat are widely used for studying the biology of breast cancer and for developing and evaluating cancer prevention and control strategies. However, very little is known about gene expression changes that are associated with experimentally induced mammary carcinogenesis. This paper reports the identification, by differential display of mRNA and molecular cloning, of seven cDNA fragments of gene transcripts overexpressed in mammary carcinomas induced by 1-methyl-1-nitrosourea. These genes included the rat homologues of human galectin-7 gene, the human/mouse melanoma inhibitory activity/bovine chondrocyte-derived retinoic acid sensitive protein gene, the mouse stearoyl-CoA desaturase-2 gene, and the mouse endo B cytokeratin/human cytokeratin-18 gene. Although each of these genes has been implicated in some aspect of carcinogenesis in other organs, this paper is the first report of their overexpression in chemically induced mammary carcinomas. Two previously uncharacterized gene transcripts were also identified. A comparison of the expression levels of several genes in mammary carcinomas with those in the normal mammary gland tissue of virgin rats, mid-stage pregnant rats, and of day 1 postpartum lactating dams indicated that the overexpression of several genes observed in mammary carcinomas could not be accounted for by either a difference in the mammary epithelial content between mammary carcinoma and normal mammary tissue or by mammary epithelium–specific proliferation associated with pregnancy. Several genes were also overexpressed in rat mammary carcinomas induced by 7,12-dimethylbenz[*a*]anthracene but not in azoxymethane-induced rat colon adenocarcinomas. The genes identified in this study may therefore represent mammary carcinoma-specific molecular markers that may be helpful in investigations of mammary carcinogenesis and its prevention. *Mol. Carcinog. 20:204–215, 1997.*

Key words: mammary carcinogenesis; cancer markers; differential display; molecular cloning; gene expression

INTRODUCTION

The induction of mammary carcinogenesis in virgin female rats by administration of l-methyl-l-nitrosourea (MNU) or 7-12-dimethylbenz[*a*]anthracene (DMBA) is the most widely used model of investigating breast carcinogenesis in women [1–4]. Comparisons of the similarities and differences in mammary carcinogenesis in rats and humans have been reported, and the findings obtained by using these models have been extensively reviewed [3,4]. One aspect of carcinogenesis that has received limited attention, however, is the characterization of the pathogenetic changes associated with experimentally induced mammary carcinogenesis in the rat and comparison of these changes with those that occur during human carcinogenesis.

Cancer is the result of mutation or misregulation of normal cellular genes. The outcome of a gene mutation or misregulated gene expression, by and large, is not immediate; rather, the development of breast cancer and other types of cancer results from accumulation of pathogenetic events [5–7]. A pathogenetic event is a general term for the many different types of DNA damage that can lead to gene amplification, point mutation, rearrangement, and deletion. The complexity of the multistep pathogenetic process makes the task of understanding the development of specific cancers, and particularly the role of specific genes, quite difficult. Nevertheless, patterns of specific gene mutations are emerging and being correlated with the development of particular cancers [6–8]. The recent discovery and cloning of breast cancer susceptibility genes *BRCA-1* and *BRCA-2* [9–11] highlight the significant progress that has been made in understanding the role of specific genes in hereditary breast cancer, which accounts for only a minor fraction (<5%) of breast cancer cases. With respect to specific gene mutations in chemically induced mammary carcinomas in the rat, Ha-*ras* codon 12 mutations (in the MNU model) and codon 61 mutations (in the DMBA model) are the only well-characterized oncogene mutations in these models,

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Abbreviations: MNU, 1-methyl-1-nitrosourea; DMBA, 7,12-dimethylbenz[a]anthracene; DD, differential display; AOM, azoxymethane; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EST, expressed sequence tag; MIA, melanoma inhibitory activity; CD-RAP, chondrocyte-derived retinoic acid–sensitive protein; SCD-2, stearoyl-CoA desaturase-2; RT, reverse transcriptase; TPS, tissue polypeptide-specific.

and each is found in only some of the carcinomas induced in the model [12–15]. Little is known about other pathogenetic events in these models. One measurable consequence of these events is overexpression of particular genes. In fact, overexpression of specific genes such as *HER-2/neu* and *myc* has been associated with development of breast cancer [16–19].

As a step towards filling this information void, this study was undertaken to identify gene expression changes, by using differential display (DD) of mRNA [20] and molecular cloning, in mammary carcinomas induced in female rats by MNU injection. The underlying hypothesis was that changes in gene expression would be observed in mammary carcinomas relative to mammary tissues in three distinct physiological states: the virgin mammary gland, the fully differentiated day 1 postpartum lactating mammary gland, and the rapidly proliferating mammary gland during mid-stage pregnancy. Here, we report the identification and cloning of seven cDNA fragments of overexpressed genes in rat mammary carcinomas.

MATERIALS AND METHODS

Chemical Carcinogenesis and Tissue Sources

The rat mammary carcinomas used for the DD of mRNA were excised from MNU-injected rats treated with a short-term carcinogenesis protocol [21]. Briefly, female Sprague Dawley rats (Taconic Farms, Germantown, NY) were given an intraperitoneal injection of MNU (50 mg/kg body weight) at 21 d of age. One rat was found to have multiple mammary tumors. This animal was killed 2 mo after the injection, and three mammary adenocarcinomas, uninvolved mammary tissue, and a kidney were immediately excised and frozen in liquid nitrogen. Tumors, tissue samples, and organs were similarly obtained from other rats. Day 1 postpartum lactating mammary gland tissue was pooled from several dams. To obtain mammary tissues from mid-stage pregnant rats, female rats that had been with a male rat for 12 d were killed, and mammary tissues were immediately excised and frozen in liquid nitrogen. The uterus of each rat was inspected to confirm the approximate stage of pregnancy based on the size of the fetuses. Two females were determined to be in the mid-stage of pregnancy; thus, mammary tissues from these animals were used in this study.

To determine the relevance of the genes identified in this short-term model to the conventional MNU carcinogenesis model in which the carcinogen is administered at 50 d of age, mammary carcinomas were also obtained by intraperitoneal injection of MNU into rats at 50 d of age [22], and these carcinomas were analyzed for gene expression by Northern blot analysis. DMBA-induced rat mammary carcinomas were obtained from Dr. Clement Ip (Roswell Park Cancer Institute, Buffalo, NY) and were examined to determine the mRNA levels of several overexpressed genes identified in the MNU-induced mammary carcinomas. In addition, two rat colon adenocarcinomas induced by azoxymethane (AOM) and matching uninvolved colon tissue were obtained from Dr. Dennis Ahnen (University of Colorado Health Sciences Center, Denver, CO) and were analyzed for gene expression by Northern blotting.

RNA Isolation

Total RNA was extracted from carcinomas and tissues by acidic phenol extraction with RNAzolB reagent (Tel-Test, Inc., Friendswood, TX). For DD, the total RNA preparations were further digested with RNase-free DNase (GenHunter Corporation, Nashville, TN) to remove contaminating genomic DNA. For cDNA library construction, poly(A)+ mRNA was enriched by oligo (dT)-cellulose column chromatography.

DD

DD of mRNA [20] was performed with the RNAimage kit (GenHunter Corporation) according to the manufacturer's instructions with the following modification. In Denver, CO, where the altitude is 5280 ft, the optimal annealing temperature for polymerase chain reaction (PCR) was determined to be 42°C. The strategy used for DD is illustrated in Figure 1. The PCR products (labeled with [α-^{32}P]dCTP) from three mammary adenocarcinomas, the uninvolved mammary tissue, and the kidney were compared side by side on sequencing gels and detected by autoradiography. Only bands present in carcinoma lanes but absent in both the mammary tissue and kidney lanes were excised and reampli-

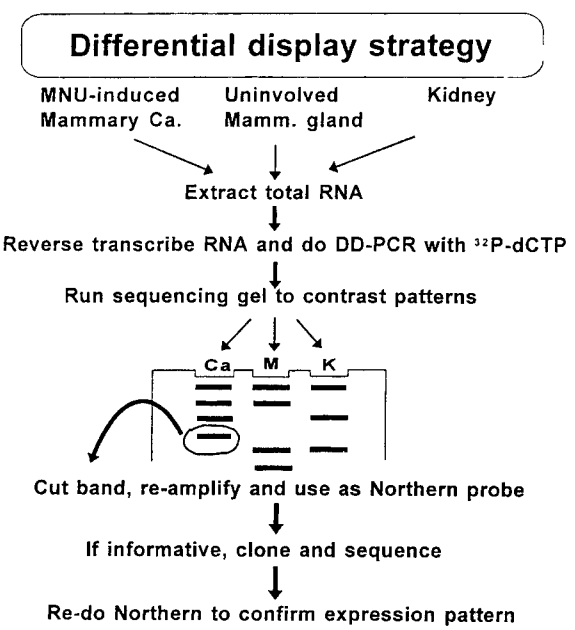

Figure 1. Schematic illustration of the DD strategy used in this study.

fied by PCR. The PCR products were separated on a low-melting-point agarose gel, and DNA bands of the expected size were eluted with a Wizard PCR DNA purification kit (Promega Corp., Madison, WI). The gel-purified PCR DNA products were used as templates to generate ^{32}P-labeled probes for Northern blot detection of gene expression on a screening panel of RNA preparations. This panel included the kidney, the three mammary carcinomas, and the uninvolved mammary tissue that were used for the original DD and two liver samples. In addition, the day 1 postpartum mammary tissue was included in this screening panel as a control for mammary epithelial cell number. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was hybridized as an internal control for loading correction. Ethidium bromide staining of the agarose gels before the RNA was transferred to nitrocellulose membrane and hybridization for the β-actin gene also were performed in some cases to verify loading and RNA integrity.

Cloning and Sequencing

PCR bands that detected differential gene expression were cloned into pGEM-T (Promega Corp.). For each band, four clones were inoculated, and the plasmid DNA was isolated by an alkaline mini-prep procedure. At least two clones were sequenced on both strands by the dideoxy chain termination method of Sanger et al. [23] by using a kit from U.S. Biochemicals (St. Louis, MO). A commercial sequencing service (Cornell DNA Service, Ithaca, NY) also was used to confirm the sequence of a few of the clones. The cloned cDNA fragments were used as templates to generate randomly labeled probes for Northern blot analysis again to confirm that the cloned sequences corresponded to the gene transcripts originally detected by the PCR products eluted from the DD gels. A homology search was performed by using the BLASTN algorithm [24] with GenBank non-redundant (nr) databases and expressed sequence tag (EST) databases.

Cloning of Full-Length cDNA

A cDNA library was constructed with pooled poly(A)+ mRNA isolated from mammary carcinomas by using the Marathon cDNA construction kit (Clontech, Inc., Palo Alto, CA). The average length of the library inserts was 1.5 kb. Based on the sequence information obtained for each gene fragment, a gene-specific primer was synthesized (Integrated DNA Technology, Inc., Coralville, IA) as the downstream PCR primer. The gene-specific primer and a universal upstream primer that annealed to the adapter that had been ligated into the cDNA library were used for long-distance PCR with KlenTaq (a combination of Taq and Vent polymerases) (Clontech Inc.) to increase fidelity of cloning. The gel-purified PCR fragments were cloned into pGEM-T and sequenced as described above.

RESULTS

Differentially Expressed Gene Transcripts

A total of 15 primer-pair combinations were used to compare by DD the gene expression patterns of three MNU-induced mammary carcinomas with those of the uninvolved virgin mammary tissue and kidney from the same rat. In initial screening, 21 cDNA fragments were used as probes for Northern analysis, and 16 detected transcripts differentially expressed in the mammary carcinomas and uninvolved virgin or day 1 postpartum lactating mammary tissue. Seven cDNA clones were characterized in more detail and are reported here.

Representative Northern blot detection of steady-state gene transcripts in a screening panel of tissues by using cloned cDNA fragments as probes is shown in Figure 2. Several points are noteworthy. First, most of the clones detected overexpression in mammary carcinomas (lanes 2–4) in comparison with the mammary tissue (lanes 7 and 8), kidney (lane 1), and liver (lanes 5 and 6). The exceptions were clone 9, which detected low-level expression in one of two liver samples and clones 14 and 10, which detected weak expression in liver and mammary gland tissue. Second, some clones detected variable gene expression levels among mammary carcinomas (e.g., clones 1

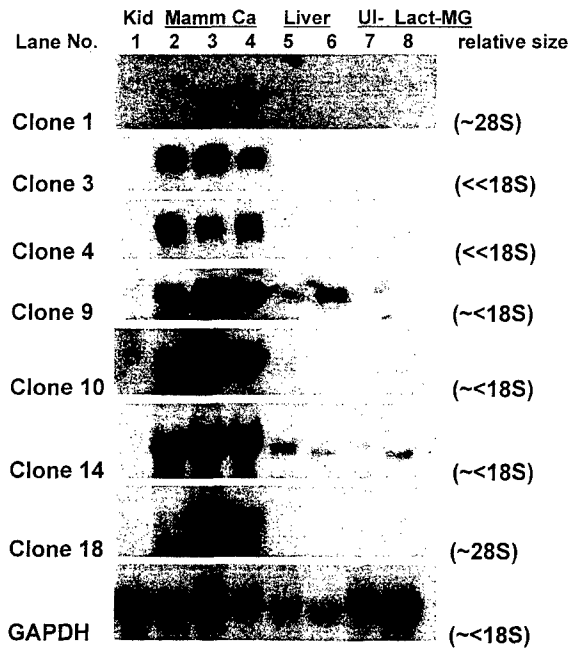

Figure 2. Northern blot analyses of gene expression detected by cloned cDNA fragment on a screening panel of RNA isolated from rat kidney (lane 1), three MNU-induced mammary carcinomas (lanes 2–4), livers (lanes 5 and 6), the uninvolved virgin mammary gland (UI, lane 7), and the day 1 postpartum lactating mammary gland (Lact-MG, lane 8). The sizes of the full transcripts relative to the 18S and 28S rRNA bands are indicated to the right of each blot. Approximately 30 μg of total RNA was loaded per lane. The *GAPDH* gene was hybridized as an internal control for loading correction.

and 18) from the same animal, whereas others detected gene expression in every carcinoma examined. Third, the overexpression of genes identified here could not be accounted for by a difference in the proportion of mammary epithelial cells between the mammary carcinoma and the mammary tissue. This was supported by the lack of or very weak gene expression in both uninvolved virgin mammary gland tissue, which is about 5% epithelial cells (lane 7), and the day 1 postpartum lactating mammary tissue, which consists primarily of secretory epithelium (lane 8).

Whether the overexpressed gene transcripts detected in the mammary carcinomas might be due to mammary epithelial-specific proliferation was addressed next. Because it is well established that during pregnancy, mammary epithelial cells undergo extensive proliferation, the expression levels of several genes (for which the full-length cDNA has been cloned, i.e., clones 3, 4, and 10) in mammary gland tissue from an early (Figure 3, lane 4) and two mid-stage (lanes 5 and 6) pregnant rats (judged by fetus size) were compared with the levels in mammary carcinomas by Northern blot analysis. Hybridization for β-casein expression confirmed the stages of pregnancy of the dams. To verify equal loading of RNA from the different tissues, the agarose gels were stained with ethidium bromide before RNA transfer and again after transfer to confirm complete transfer. *GAPDH* and β-actin were hybridized to verify RNA integrity. For clones 3 and 4, the mammary epithelial proliferation that is associated with pregnancy could not account for the overexpression observed in mammary carcinomas. However, for clone 10, mammary-specific proliferation may partially account for the overexpression observed.

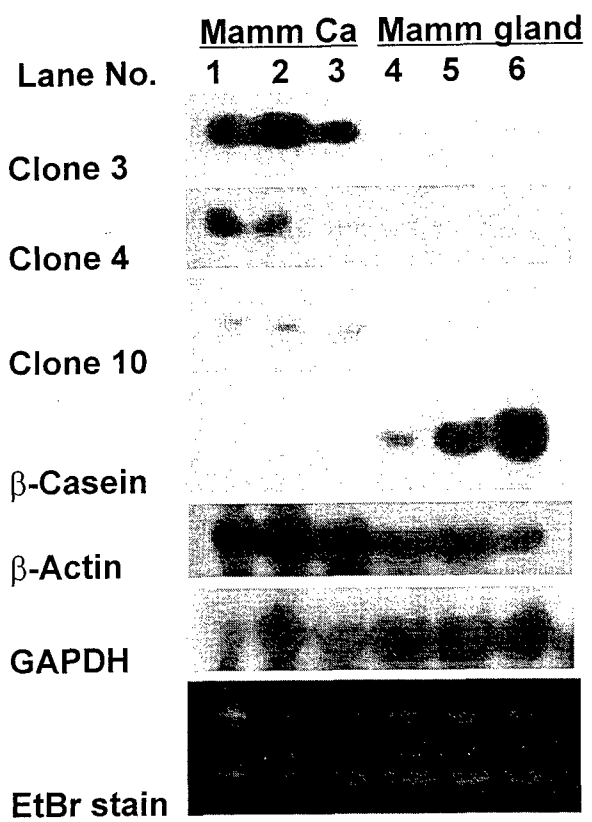

Figure 3. Northern blot analyses of expression of transcripts in mammary carcinomas (lanes 1–3) and early-stage pregnant (lane 4) and mid-stage pregnant mammary gland (lanes 5 and 6). The stage of pregnancy was determined by the size of the fetuses at necropsy and was confirmed by β-casein expression. Approximately 30 μg of total RNA was loaded per lane. Ethidium bromide (EtBr) was used to stain the agarose gel before transfer. The *GAPDH* and β-actin genes, along with the rRNA bands, served as loading and RNA integrity controls.

The sequences of the cloned gene fragments are shown in Figure 4. These sequences were submitted to GenBank in August 1996, and their accession numbers are given in Table 1. A homology search with BLASTN of the GenBank nr databases identified homologues of several known genes (Table 1). These included clone 3, human galectin-7 [25]; clone 4, human/mouse melanoma inhibitory activity (*MIA*) [26,27]/bovine chondrocyte-derived retinoic acid–sensitive protein (*CD-RAP*) [28]; clones 10 and 14 (which were identical except for five bases preceding the poly(A) tail), mouse endo B cytokeratin [29]/human cytokeratin-18 [30]; and clone 18, mouse stearoyl-CoA desaturase-2 (*SCD-2*) [31]. Comparison with the GenBank EST database revealed significant homology among clone 1, two mouse EST clones (gbAA014143 and gbW36666), and a human EST clone (gbN25349). Clone 9 had significant homology to human EST gbN35187 and to several mouse EST clones (i.e., gbW80253 and gbW82774).

Gene Expression in Rat Mammary Carcinomas Induced by Conventional MNU and DMBA Protocols

Because the mammary carcinomas used for the DD were obtained by injecting MNU into sexually immature rats at 21 d of age [21], the expression patterns of several genes were next examined in mammary carcinomas induced by MNU injection or gastric gavage of DMBA into female Sprague Dawley rats at 50 d of age (the conventional protocols). Figure 5 shows the expression patterns detected by clones 3 (galectin-7), 4 (MIA/CD-RAP), 9, and 10/14 (cytokeratin-18). Despite some variability in the expression levels among the carcinomas, these data provided strong evidence of expression of all four genes in the rat mammary carcinomas regardless of the induction protocol and the chemical carcinogen used.

Because many proteins present in carcinomas reflect derepressed expression of embryonic genes, the levels of expression of the four expressed genes were examined in RNA extracted from rat fetuses from mid-stage pregnant rats (Figure 5, lane 3). The expression levels were below the Northern blot detection limit

CLONE 1

```
                                                                                                    AAGCTTCGACTGT
1     CAAGGAACTA AAACCTTTAA AAGCATAGGC ATGCTGGCCT GAGGTAAACA CTGGTACAGT TAGAGCGGAG GCAGCAGCCC CGGTGTTCAG
91    CACAGCTTGT CTGATGTGTT TATGGCCAGA GTGCAGTATT CGGCACTGGC TAGTACCGCT GCCTGACCGA ACTCCACTGG GAAGGTTTTG
181   CTTAAACGCA CATGTTTCTT TGTTACTCCT GACCATGCTT GGATGCAGTG CCTANATTGT GGCTGCTATT TTTAGTTCGC ATCATGACCT
271   TGACTGCGGA GTCCAGCCTT ACCCTCTTAC TTGATAACAG TAGCCCTTAA ACTGCAGTGG GAAGAAAGAA GAATTTGGTA TGAAAATTGG
361   TGAGCTCTGG CACTTGAGAT AAACAAGAAG AAAGTTCCAA CTTGATGCCT TAAGCCGCTC CTGCCCGAAG CTCTGAAGGA ACTAAGTTGG
451   CAGAGATCCT TACTTTGATA CTACTGCATT TTTTGTAGAA TTGTTACATT GATAATAAAA CTTGCCTGCT TAACCT CAAAAAAAAAAAGCTT
```

CLONE 3

```
                                                                                                    AAGCTTCGACTGT
1     GATCGGGGAT GACGAGTATC TCCACTTCCA CCACCGGATG CCATCCTCTA ACGTGCGCTC AGTGGAGGTG GGCGGAGACG TGCAGCTGCA
91    TTCTGTGAAG ATCTTCTGAG CAAGGACCCA GGGGCTTGGC GAGTGGGGGT GGGGTTTCGT CAGATCGTAG AGGAGGGTTG TGGATGGCGA
181   ATAAACTGTA GCTGTAGTTC CAAAAAAAAAAAGCTT
```

CLONE 4

```
                                                                                                    AAGCTTCGACTGT
1     CAATGAGCTC AACCCACCGA TGTTATCCCT GCAGTTACCC TTCCGGTTTG GGCAAATACA GCGGCCAACT GCAAAGTGTT TTGTCCCTTT
91    GGTTTTTGGG GTGGGCATGT ACAAAGAATG TTTCACGGGT TCCTGAACCT AGCCAATTAA AGCCCTGAAT GTTGTAACGT
      CAAAAAAAAAAAGCTT
```

CLONE 9

```
                                                                                                    AAGCTTTGGTCAG
1     AGGTGCCAGA AGTGTTTTGG GTCTCAATTT GAGAGCCCTG AGTTCTCCAC AGAGAGCATT GAAAGGATGC TGAATAACCT GGTTAATTAT
91    ATTCTGCAAA GATACTACGG ACATAGGAAG ATAGCATCGA TCTTGAATGC ATCTTTGGAT GAAAAGGTGC TTTTGGGATG GGCCCCAAGA
181   ACAACATAAA ATGGGGAGGC ATGCAGTTCT TGCACCCCCA TGGAAGTGTG CCGTTGCACA GAGAGTGAGG CCACCCAGAT CCCCATCTCC
271   ATCTCCATCG CCATCACCAA AGAGCCACAG TTCCTCTCCA TCAAGGAGCC ACCCTCCTTT ACTTCAGACT GRGATTACAG GTTTTAGGAA
361   CAGGCCTTTT CAGGAAGTCA GAGAGCCCGG TGAAGCAGCA TCACCACTAT TAGTGATTTT GTCTGTCGCT GCGCTTGCCC TTATTGGTTT
451   CTTCATTAGA TAATAGGTTT ATCATCTTGT TTTAATCCCG TGGTGATCAA TAAAATGGAT GTCATTTTAA CGT
      GAAAAAAAAAAGCTT     R=A OR G (polymorphic in 2 clones)
```

CLONE 10

```
                                                                                                    AAGCTTCTCAACG
1     ACGCCCTGGA CTCCAGCAAC TCCTAGCAAA CTGCCAGAGG ACAACTACCC GTAAGGTCGT GGATGGCAAA GTGGTGTCCG AGACCAATGA
91    TACCAGAGTT CTGAGGCACT AAGGCTCAGA AGAAGGGAAC CCTTGGGGAC TGAGGGTCCA ATAAAAGTTT AGAATCCACT G
      GAAAAAAAAAAAGCTT
```

CLONE 14

```
                                                                                                    AAGCTTCTCAACG
1     ACGCCCTGGA CTCCAGCAAC TCCATGCAAA CTGCCAGAGG ACAACTACCC GTAAGGTCGT GGATGGNAAA GTGGTGTCCG AGACCAATGA
91    TACCAGAGTT CTGAGGCACT AAGGCTCAGA AGAAGGGAAC CCTTGGGGAC TGAGGGACCA ATAAAAGTTT AGAATC CAAAAAAAAAAAGCTT
```

CLONE 18

```
                                                                                                    AAGCTTTGGTCAG
1     GGTAGGAGAT CTGAGATGCC GTGGACTTTG CAGAARAGAT TTTGTGCAGC AACGCAGAAG CCTGGCTGTG TTAAGATTGG CTGTACAGGA
91    TAGCGAACTG TGGTTGGAGG GCACAGTCTT CCCGTGTTAG TTAAATAGAG AGGCTTTAGG ACGTCTCTGC GCCCACTTGA AGGATGCCTT
181   ATCCTTTAGC TGTTCGCTAA AATAGAATCC TTGAGAGATG ACAATATATT TGCTGCCTGT CGCCCTATTC CTTGGAGAGG CCTTTTGAAT
361   GGTTTTAATT CCACTGGTTA TCCGGTGCCC ACTTAATCAA ATGT GAAAAAAAAAAAGCTT R=A OR G (polymorphic in 2 clones)
```

Figure 4. Nucleotide sequences of cloned cDNA fragments. The lightly-underlined sequences are the primers used for DD of mRNA. Translation termination codons for known genes are heavily underlined. Canonical polyadenylation signals are in bold.

Table 1. Gene Fragments Cloned by DD of mRNA from MNU-induced Rat Mammary Carcinomas

Clone no.	Fragment size (bp)*	GenBank accession no.	Homologues identified by BLASTN search	% homology†
1	526	U67990	Mouse EST AA014143	234/250 = 94%
			Mouse EST W36666	131/173 = 76%
			Human EST N25349	138/178 = 78%
3	200	U67883	Human galectin-7 U06643&L07769	91/124 = 73%
4	170	U67884	Human melanoma inhibitory activity, MIA, X75450	61/105 = 58%
			Mouse MIA X94322	96/106 = 90%
9	523	U67991	Mouse EST W82774	260/318 = 82%
			Mouse EST W80253	143/166 = 86%
10	171	U67992	Mouse endo B cytokeratin, M11686	102/116 = 87%
			Human cytokeratin-18, X12876, M26325	64/82 = 78%
14	166	U67992	Same as clone 10, except missing 5 bp proceeding poly (A)	
18	314	U67995	Mouse stearoyl-CoA desaturase-2, U67995	194/239 = 81%

*Excluding DD-PCR primer sequences.
†Homology was calculated based on the sum of stretches of DNA sequences matched by BLASTN with GenBank nr or EST databases.

for all four genes. However, a low level of expression in the fetuses was likely because the full-length rat *MIA/CD-RAP* cDNA (clone 4) was successfully cloned by reverse transcriptase (RT)-PCR from this RNA extract (see "Cloning of Full-length cDNA" below).

Expression Patterns in Non-mammary Tissues and Colon Adenocarcinomas

The expression patterns detected by clones 3 (galectin-7), 4 (*MIA/CD-RAP*), 10/14 (cytokeratin-18), and 18 (*SCD-2*) in nonmammary tissues are shown in Figure 6A. Clear-cut mammary carcinoma–specific expression was detected by clone 4 when compared with 11 other non-mammary tissues. Modest expression was detected by clone 3 in the stomach (about one-tenth the expression level in carcinomas). Strong expression was detected by clone 18 in the brain. Clones 10/14 (cytokeratin-18) detected weak expression in liver and in organs with epithelial linings, such as stomach, intestine, colon, and lung.

The expression levels of clones 3 (galectin-7) and 4 (*MIA/CD-RAP*) in two rat colon adenocarcinomas induced by AOM and the matching uninvolved colon tissues were determined by Northern blot analysis (Figure 6B). There was no detectable signal for either gene in either the cancerous or uninvolved

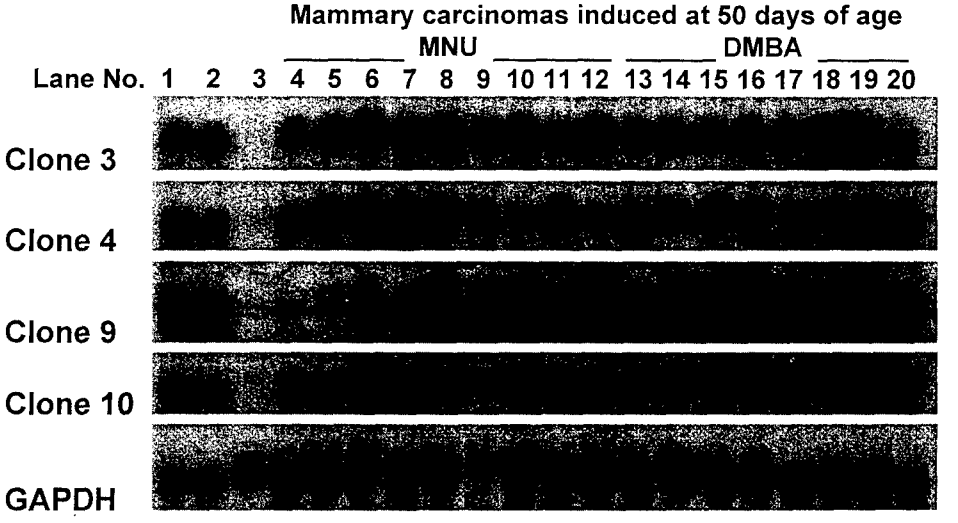

Figure 5. Northern blot analyses of expression of galectin-7 (clone 3), *MIA/CD-RAP* (clone 4), cytokeratin-18 (clones 10/14), and clone 9 in rat mammary carcinomas induced by MNU or DMBA administered when the rats were 50 d of age. Lanes 1 and 2, mammary carcinomas used for DD; lane 3, mid-term rat fetuses; lanes 4–12, mammary carcinomas induced by an intraperitoneal injection of MNU at 50 d of age; lanes 13–20, mammary carcinomas induced by gastric gavage of DMBA at 50 d of age. Approximately 30 μg of total RNA was loaded per lane. The *GAPDH* gene was hybridized as an internal control for loading correction.

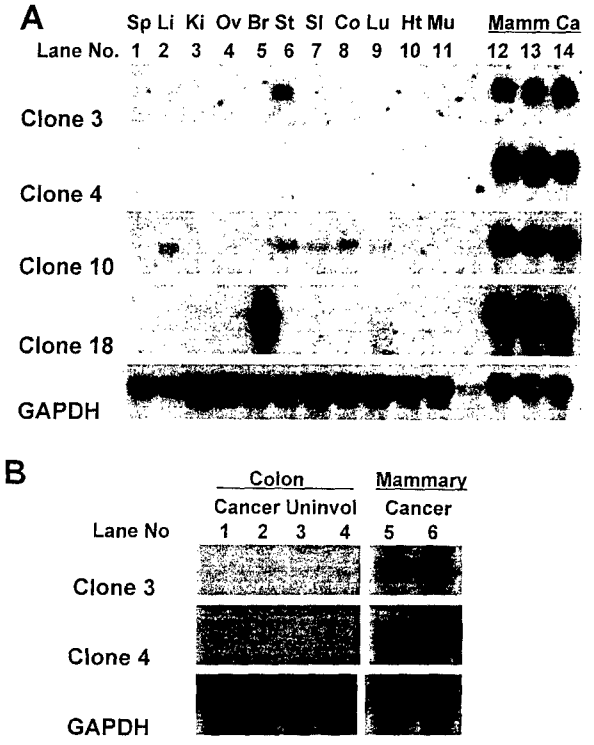

Figure 6. (A) Northern blot analyses of expression of galectin-7 (clone 3), *MIA/CD-RAP* (clone 4), cytokeratin-18 (clones 10/14), and *SCD-2* (clone 18) in rat non-mammary tissues. The tissues examined were spleen (lane 1), liver (lane 2), kidney (lane 3), ovary (lane 4), brain (lane 5), stomach (lane 6), small intestine (lane 7), colon (lane 8), lung (lane 9), heart (lane 10), and leg muscle (lane 11). Lanes 12–14, mammary carcinomas induced by MNU by using the short-term protocol. (B) Northern blot analyses of expression of galectin-7 (clone 3) and *MIA/CD-RAP* (clone 4) in AOM-induced rat colon adenocarcinomas (lanes 1 and 2) and matching uninvolved colon tissue (lanes 3 and 4). Two MNU-induced rat mammary adenocarcinomas (lanes 5 and 6) were analyzed on the same blots for comparison. Approximately 30 μg of total RNA was loaded per lane. The *GAPDH* gene was hybridized as an internal control for loading correction.

colon tissue, whereas the mammary carcinomas on the same blots showed the expected bands.

Cloning of Full-length cDNA

The full-length sequences were obtained for rat galectin-7 (Figure 7, GenBank Accession No. U67883) and *MIA/CD-RAP* (Figure 8, Accession No. U67884). Full-length rat galectin-7 was 76% homologous to human galectin-7 at the nucleotide level and shared 72% identity and 84% positivity (i.e., similar charge characteristics) at the predicted amino-acid level (Figure 7).

The rat *MIA/CD-RAP* cDNA was also cloned from both a mid-term and a full-term fetal rat RNA preparation by RT-PCR, and the sequences were compared with that derived from the mammary carcinomas. No mutation was detected in the coding region of the cDNA cloned from the carcinomas. The deduced rat MIA/CD-RAP protein sequence was aligned with the human sequence and those of other species in Figure 8. Despite some variability in the signal peptide region (the first 22–24 amino acids) among species, the predicted mature MIA proteins (i.e., minus the secretory peptide) were highly conserved (94% identity with both human and mouse MIA and 90% identity with bovine CD-RAP). The effect of expression of full-length cDNAs of galectin-7, *MIA/CD-RAP*, and other genes on mammary epithelial cells is currently being evaluated.

DISCUSSION

This paper reports the identification and cloning of seven cDNA fragments of genes whose overexpression appeared to be specifically associated with chemically induced rat mammary carcinomas. A comparison of the expression patterns of several of these genes in mammary carcinomas with genes in uninvolved virgin mammary tissue, day 1 postpartum lactating mammary tissue, and mammary tissue of mid-stage pregnant dams indicated that the overexpression observed in mammary carcinomas could not be accounted for by a difference in the epithelial content of the mammary carcinoma and the mammary tissue or by mammary epithelial–specific proliferation associated with pregnancy (Figures 2 and 3). That the overexpressed gene transcripts identified in MNU-induced rat mammary carcinomas were also detected in DMBA-induced carcinomas (Figure 5) indicates a commonality of these models with respect to the overexpression of these genes and may imply that the products of these genes play a role in mammary carcinogenesis in both chemically induced breast cancer models. Among 11 organs and tissues examined by Northern blot analysis, the expression of most of the genes was restricted to the mammary carcinomas (Figure 6A). This was further supported by the lack of expression of galectin-7 and *MIA/CD-RAP* in AOM-induced rat colon adenocarcinomas (Figure 6B). Taken together, these results support the hypothesis that at least some of these genes may serve as specific markers of mammary carcinogenesis. Whether the altered expression of these genes plays a causal role in mammary carcinogenesis is currently under investigation.

None of the known homologues of the genes identified in this study has previously been examined in chemically induced rat mammary carcinomas. However, each of these known genes or gene families has been implicated in some aspect of carcinogenesis in other organ sites. The relevant information is discussed below.

Whereas little is known about the role of galectin-7 in normal physiology or carcinogenesis, other members of the galectin family of proteins, which are characterized by the common property of binding to galactosyl moieties with conserved primary structural features [32,33], have been examined as cancer-specific markers in several organ sites [34–39]. Galectin-4 was recently been shown to be expressed

CLONE 3 RAT GALECTIN-7

```
                                                        5'-TTGC CGTGCCAGCC

15      ATG TCT GCC ACC CAT CAC AAG ACC CCT CTG CCT CAG GGT GTC CGC
1       Met Ser Ala Thr His His Lys Thr Pro Leu Pro Gln Gly Val Arg

60      CTG GGC ACC GTC ATG AGA ATT CGA GGC GTG GTC CCT GAC CAG GCT
16      Leu Gly Thr Val Met Arg Ile Arg Gly Val Val Pro Asp Gln Ala

105     GGC AGG TTC CAT GTA AAC CTG CTA TGC GGC GAG GAG CAA GAG GCA
31      Gly Arg Phe His Val Asn Leu Leu Cys Gly Glu Glu Gln Glu Ala

150     GAC TGC GCC CTG CAC TTT AAC CCG AGG CTG GAC ACA TCC GAG GTT
46      Asp Cys Ala Leu His Phe Asn Pro Arg Leu Asp Thr Ser Glu Val

195     GTC TTC AAC ACC AAA CAG CAA GGC AAA TGG GGC CGT GAG GAG CGG
61      Val Phe Asn Thr Lys Gln Gln Gly Lys Trp Gly Arg Glu Glu Arg

240     GGC ACC GGC ATC CCC TTC CAG CGT GGG CAG CCC TTT GAA GTG CTC
76      Gly Thr Gly Ile Pro Phe Gln Arg Arg Gln Pro Phe Glu Val Leu

285     ATC ATC ACC ACA GAG GAA GGC TTC AAG ACT GTG ATC GGG GAT GAC
91      Ile Ile Thr Thr Glu Glu Gly Phe Lys Thr Val Ile Gly Asp Asp

330     GAG TAT CTC CAC TTC CAC CAC CGG ATG CCA TCC TCT AAC GTG CGC
106     Glu Tyr Leu His Phe His His Arg Met Pro Ser Ser Asn Val Arg

375     TCA GTG GAG GTG GGC GGA GAC GTG CAG CTG CAT TCT GTG AAG ATC
121     Ser Val Glu Val Gly Gly Asp Val Gln Leu His Ser Val Lys Ile

420     TTC TGA GCAAGGACCC AGGGGCTTGG CGAGTGGGGG TGGGGTTTCG TCAGATCGTA
136     Phe Stop

476     GAGGAGGGTT GTGGATGGCG AATAAACTGT AGCTGTAGTTC C poly (A) -3'
```

```
Rat           1  MSATHHKTPL PQGVRLGTVM RIRGVVPDQA GRFHVNLLCG EEQEADCALH
                 ||   ||+ | |+|+| |||+ ||||+|| +|  ||||||||| ||| +| |||
Human [25]    1  MSNVPHKSSL PEGIRPGTVL RIRGLVPPNA SRFHVNLLCG EEQGSDAALH

Rat          51  FNPRLDTSEV VFNTKQQGKW GREERGTGIP FQRGQPFEVL IITTEEGFKT
                 |||||||||| |||+|+|| | |||||| |+| |||||||||| || +++|||
Human        51  FNPRLDTSEV VFNSKEQGSW GREERGPGVP FQRGQPFEVL IIASDDGFKA

Rat         101  VIGDDEYLHF HHRMPSSNVR SVEVGGDVQL HSVKIF
                 |+|| +| ||  ||+| + ||  ||||||||  ||+||
Human       101  VVGDAQYHHF RHRLPLARVR LVEVGGDVQL DSVRIF
```

Figure 7. Sequence of full-length cDNA of rat galectin-7 and alignment of its deduced amino-acid sequence with the human homologue [25]. Translation initiation and termination codons are in bold type. Vertical lines indicate amino-acid identity, and plus signs indicate similar charge characteristics for the encoded amino acids. The GenBank accession number is U67883.

CLONE 4 RAT MIA/CD-RAP

```
                                                        5'-TT GAAGTCCATG

 13    ATG GTG TGC TCC CCA GTG CTC CTT GGT ATT GTC ATC TTG TCT GTT
  1    Met Val Cys Ser Pro Val Leu Leu Gly Ile Val Ile Leu Ser Val

 58    TTT TCA GGC CTC AGC AGG GCT GAT CGA GCC ATG CCC AAG CTG GCT
 16    Phe Ser Gly Leu Ser Arg Ala Asp Arg Ala Met Pro Lys Leu Ala

103    GAC CGG AAG CTG TGT GCA GAT GAG GAG TGT AGC CAT CCT ATC TCC
 31    Asp Arg Lys Leu Cys Ala Asp Glu Glu Cys Ser His Pro Ile Ser

148    ATG GCT GTG GCC CTT CAG GAC TAC GTG GCC CCT GAT TGC CGC TTC
 46    Met Ala Val Ala Leu Gln Asp Tyr Val Ala Pro Asp Cys Arg Phe

193    TTG ACT ATA TAC AGG GGC CAA GTG GTA TAT GTC TTC TCC AAG TTG
 61    Leu Thr Ile Tyr Arg Gly Gln Val Val Tyr Val Phe Ser Lys Leu

238    AAA GGC CGT GGA CGG CTT TTC TGG GGA GGC AGT GTG CAG GGA GAT
 76    Lys Gly Arg Gly Arg Leu Phe Trp Gly Gly Ser Val Gln Gly Asp

283    TAC TAT GGA GAC CTG GCA GCC CAC CTG GGC TAT TTC CCC AGT AGC
 91    Tyr Tyr Gly Asp Leu Ala Ala His Leu Gly Tyr Phe Pro Ser Ser

328    ATT GTC CGG GAG GAC CTG ACT CTG AAA CCT GGC AAA GTC GAT ATG
106    Ile Val Arg Glu Asp Leu Thr Leu Lys Pro Gly Lys Val Asp Met

373    AAG ACA GAT GAA TGG GAT TTC TAC TGT CAA TGA          GCTCAACCCA
121    Lys Thr Asp Glu Trp Asp Phe Tyr Cys Gln Stop

416    CCGATGTTAT CCCTGCAGTT ACCCTTCCGG TTTGGGCAAA TACAGCGGCC AACTGCAAAG
476    TGTTTTGTCC CTTTGGTTTT TGGGGTGGGC ATGTACAAAG AATGTTTCAC GGGTTCCTGA
536    ACCTAGCCAA TTAAAGCCCT GAATGTTGTA ACGTC poly (A)-3'
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This study          MVCSPVLLGI VILSVFSGLS RADRAMPKLA DRKLCADEEC SHPISMAVAL
Rat CD-RAP [28]                (partial)              LCADEEC SHPISVTVAL
Mouse MIA  [26]     MVWSPVLLGI VVLSVFSGPS RADRAMPKLA DWKLCADEEC SHPISMAVAL
Cow CD-RAP [28]     MAWSLVFLGV VLLSAFPGPS AGGRPMPKLA DRKMCADEEC SHPISVAVAL
Human MIA  [26,27]  MARSLVCLGVIILLSAFSGPG VRGGPMPKLA DRKLCADQEC SHPISMAVAL

This study          QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAAHLG
Rat CD-RAP          QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAAHLG
Mouse MIA           QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGG YYGDLAARLG
Cow CD-RAP          QDYVAPDCRF LTIHQGQVVY IFSKLKGRGR LFWGGSVQGD YYGDGAARLG
Human MIA           QDYMAPDCRF LTIHRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAARLG

This study          YFPSSIVRED LTLKPGKVDM KTDEWDFYCQ
Rat CD-RAP          YFPSSIVRED LTLKPGKVDM KTDE
Mouse MIA           YFPSSIVRED LNSKPGKIDM KTDQWDFYCQ
Cow CD-RAP          YFPSSIVRED QTLKPAKTDV KTDIWDFYCQ
Human MIA           YFPSSIVRED QTLKPGKVDV KTDKWDFYCQ
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Figure 8. Sequence of full-length cDNA of rat *MIA/CD-RAP* and alignment of its deduced amino-acid sequence with homologues from mouse [26], human [26,27], and cow [28] and the reported rat partial sequence [28]. The translation initiation and termination codons are in bold type. The predicted cleavage site for the signal peptide is between amino-acid residues 22 and 23. The GenBank accession number is U67884.

in ductal carcinoma in situ and invasive breast carcinomas but was not expressed in morphologically normal mammary epithelium [40]. Galectin-3 has been reported to protect cells against apoptosis [41], whereas galectin-1 has been implicated as a mediator of T-cell apoptosis [42]. Although each type of galectin may have specific biological functions, their common galactosyl-binding property [32,33] suggests that they may be involved in mediating cell-cell recognition and cell-matrix interactions, processes that are being increasingly implicated in the regulation of cell fates and in the maintenance of tissue-size homeostasis. Indeed, many publications support this possibility [43–48].

The human MIA protein was purified from the conditioned medium of a slow-growing melanoma cell line [26]. Molecular cloning confirmed that the gene product is a secretory protein with inhibitory activity against melanoma cell growth in culture [26]. The expression of this gene appears to be restricted to malignant melanomas [26,27] but was recently detected in cultured bovine chondrocytes as coding for a retinoic acid–sensitive protein (CD-RAP) and in fetal rodent skeletal cartilage tissues [28]. Recent data showed that MIA/CD-RAP binds to fibronectin, laminin, and tenascin [49]. In that study, it was hypothesized that such an activity could interfere with cell attachment to these matrix proteins through specific integrins and thus be involved in tumor invasion and metastasis. It is conceivable that in chemically induced mammary carcinogenesis, MIA/CD-RAP may play a similar role in tumor cell/extracellular matrix interactions. The fact that retinoic acid is a morphogen in embryogenesis and that retinoic acid represses the expression of MIA/CD-RAP in chondrocytes [28] suggests that this gene has a role in morphogenesis, tissue remodeling, and differentiation. Our sequence comparison between *MIA/CD-RAP* cloned from mammary carcinomas and from rat fetuses did not reveal any mutation in the coding region, supporting a role for gene overexpression rather than specific gene mutation in chemically induced rat mammary carcinogenesis.

Endo B cytokeratin/cytokeratin-18 and its partner endo A cytokeratin/cytokeratin-8 are extremely early embryonic genes detectable at the four- or eight-cell stage of embryonic development in the mouse [50,51]. In adult tissue, low-level gene expression, if any, is restricted to simple epithelium [52]. The low level of expression in stomach, small intestine, colon, and lung detected in this study (Figure 6A) is consistent with these findings. Clinically, overexpression of cytokeratin-18 has been observed in many types of cancer arising from simple epithelium and has been used as a diagnostic for metastatic disease [53–59]. In fact, tissue polypeptide–specific (TPS) antigen has been advocated over the past two decades as a serum tumor marker, but it was a long time before it was proven that these proteins in the serum are related to cytokeratin fragments [58]. For instance, the TPS test was based on detection of fragments of cytokeratin-18, and the TPA(cyk) test was based on detection of fragments of both cytokeratin-18 and its partner cytokeratin-8 [58]. Although a recent study showed that at the time of diagnosis of metastatic breast disease 86% of the serum values for TPS were above the upper reference value [59], the clinical experience described above suggests that cytokeratin-18 is probably not a mammary-specific cancer marker. Nevertheless, overexpression of cytokeratin-18 may play an important role in some aspect of carcinogenesis, as indicated by the observation that enforced overexpression of both cytokeratin-8 and cytokeratin-18, which are partners for assembly into intermediate filaments, confers multiple drug resistance to mouse fibroblasts in culture [60].

The *SCD-2* (clone 18) gene was initially cloned from mouse 3T3-Li cells induced to differentiate into adipocytes [31]. Under normal feeding conditions (e.g., a diet containing unsaturated triacylglycerol), *SCD-2* is expressed very strongly in mouse brain; weakly in lung, kidney, and adipose tissue; and below the detection limit in heart, spleen, and liver [31]. In contrast, the related *SCD-1* gene is expressed very strongly in the adipose tissue and was below the detection limit in other tissues under the same feeding conditions [31]. The *SCD-2* transcript is primarily localized in oligodendrocytes in the brain [61]. The size of the transcript and the tissue distribution profile of the gene detected by clone 18 in our study (Figures 2 and 6A) are consistent with this clone being the rat homolog of *SCD-2*. The products of SCDs are thought to play a key regulatory role in unsaturated fatty acid biosynthesis. The presence of *SCD-2* in the brain oligodendrocytes [31,61] and its resistance to induction by starvation and refeeding of a triacylglycerol-free diet [31] support a role for this gene product in myelination of critical brain cells. The overexpression of *SCD-2* we observed in mammary carcinomas may indicate altered lipid metabolism in mammary carcinoma cells.

In summary, seven cDNA fragments were identified that detected gene overexpression in chemically induced rat mammary carcinomas. This study provides important leads in an area that has received limited attention despite the extensive use of the MNU- and DMBA-induced mammary carcinogenesis model systems. However, many questions need to be addressed to validate these genes as specific markers of mammary carcinogenesis. These questions include (i) What specific cell type or types within mammary carcinomas express these gene transcripts? (ii) Are the protein products of these genes also overexpressed? (iii) Is the gene expression observed specific to chemically induced mammary carcinogenesis? and (iv) At what stage of mammary carcinogenesis can the expression be detected? In situ detection approaches as well as the more conven-

tional methodologies described in this paper will be used to address these issues. We note that the short-term model system developed by Thompson et al. [21] permits the investigation of these questions in intraductal hyperplastic lesions and ductal carcinomas in situ as well as frank mammary carcinomas. Carcinomas in this model system have also been observed to be locally invasive and to metastasize to the lung. Work is in progress to evaluate the role or roles of these genes in the multistep process of mammary carcinogenesis.

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SHORT COMMUNICATION

Pathogenetic characterization of 1-methyl-1-nitrosourea-induced mammary carcinomas in the rat

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The induction of mammary carcinogenesis in the rat by 1-methyl-1-nitrosourea (MNU) is widely used in experimental breast cancer research. In the experiments reported, the Ha-*ras* codon 12 (*ras*12) mutation (GGA→GAA) was used as a molecular marker to address issues of the clonality of carcinomas induced, pathogenetic independence among multiple carcinomas within the same animal and topographic distribution of mutant *ras*12 carcinomas in different mammary gland chains. In order to determine whether the frequently observed morphologically distinguishable lobules within carcinomas originate from the coalescence of independent lesions or whether cancerous cells within a carcinoma share a common origin, 44 randomly selected MNU-induced mammary carcinomas were genotyped for two to four lobules each for the *ras*12 mutation. A total of 43 carcinomas out of 44 (97.7%) had concordant *ras*12 genotypes among the multiple sites within each tumor, which is consistent with the latter possibility. Next, it was observed that as carcinoma multiplicity increased, the discordance rate of *ras*12 genotypes among multiple carcinomas within the same animal increased in a manner that was in excellent agreement with the expected discordance rate based on an assumption of no pathogenetic association among carcinomas. Furthermore, a significant difference was observed in the occurrence of mutant *ras*12 carcinomas between the cervical-thoracic and the abdominal-inguinal mammary glands in that three times as many carcinomas were mutant in the former as in the latter glands, whereas the occurrence of wild-type carcinomas was approximately the same in both regions. Taken together, the data are consistent with (i) carcinomas induced by MNU and detected by palpation are monoclonal in origin, (ii) independently-initiated cells emerge as distinct mammary carcinomas in the same animal, and (iii) the anatomical location of the gland may affect the prevalence of mammary carcinomas that harbor a mutant *ras*12.

The 1-methyl-1-nitrosourea (MNU*)-induced rat mammary carcinogenesis model (1) has contributed significantly to the current understanding of the biology of breast cancer and to potential approaches for its prevention. Major attributes of this model include that the proportion of mammary carcinomas that are ovarian-hormone dependent is similar to that observed in the human disease; that the carcinomas induced are aggressive and locally invasive; and that there is a clear operational distinction between the initiation and promotion stages of the disease process based on the action of MNU as a direct methylating agent (1–4). This latter feature of the model is often exploited to study effects of cancer preventive agents or risk factors on the promotion and progression stages of mammary carcinogenesis. Technical improvements since its original publication have made this model easier to implement and more reproducible (2,5,6). For example, Thompson and coworkers (5,6) have examined this model with respect to the route of carcinogen administration and have found that a single dose of MNU given intraperitoneally (i.p.) or subcutaneously (s.c.) was as effective as when it was given by intravenous (i.v.) injection, the method of administration originally reported (1). When MNU was administered by i.p. injection, smaller coefficients of variation in the number of carcinomas per rat were observed, an improvement the authors attributed to the consistent manner and the ease with which the MNU was delivered (6). The work reported here was based on the induction of mammary carcinogenesis by i.p. administration of MNU to female Sprague–Dawley rats at 50 days of age.

The pathogenetic characteristics of this experimental model of breast cancer are being defined with the use of molecular techniques. One of the identifiable somatic genetic changes is a GGA→GAA transition in Ha-*ras* proto-oncogene codon 12 (*ras*12) in a percentage of the carcinomas (7–9). Numerous studies have indicated that this mutation is an early initiating event (9–11) probably as a result of methylation of the guanine nucleosides (12,13), although some data have suggested that there might be a low frequency of spontaneous mutation of this codon in mammary epithelial cells (14). The early nature of the *ras*12 mutation in MNU-induced mammary carcinogenesis could therefore mark the initiated cells and their resultant carcinomas into two pathogenetic subpopulations, i.e. those with a mutant *ras*12 and those with a wild-type *ras*12 gene. Taking advantage of this mutation as a molecular marker, we addressed the following questions in order to gain further insights concerning the biology of the disease process in this model system:

1. Do the different morphologically discernible lobules that are frequently observed within mammary carcinomas (see examples in Figure 1A) indicate either that they arise from a coalescence of independent lesions (Figure 1B, Scheme 1) or that these lobules result from morphological diversification of clonally derived cells during tumor progression (Figure 1B, Scheme 2)?
2. Do multiple carcinomas within the same animal share the same pathogenetic characteristics such as *ras*12 mutation or do independently initiated foci of cells develop into distinct carcinomas?
3. Is the prevalence of mutant *ras*12 in carcinomas within an animal best modeled as a stochastic process or is there a bias based on the topographic location of the mammary gland from which a carcinoma arises?

Two animal experiments were conducted to provide the

*Abbreviations: MNU, 1-methyl-1-nitrosourea; BW, body weight; H&E, hematoxylin and eosin; C-T, cervical-thoracic; A-I, abdominal-inguinal.

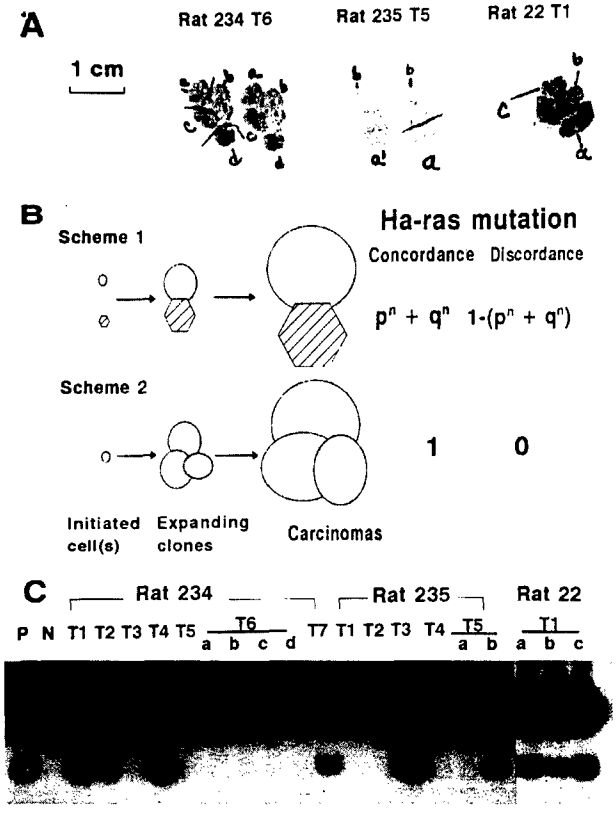

Fig. 1. (**A**) Examples of the gross appearance of tumor sections (H&E) on thin plastic slides. Tumors, especially large ones, were often observed to be made up of morphologically discernible lobules. The horizontal bar represents 1 cm in length. Sample code key: Rat234T6a, Rat #234, tumor #6, sampled site a. Lower case letters indicate sites from which tissue was sampled for *ras*12 genotyping. (**B**) Schematic illustration of multiple independent origins (polyclonality, Scheme 1) and a common origin of cancerous cells within a carcinoma (monoclonality, Scheme 2). (**C**) PCR-RFLP analysis of *ras*12 genotype in carcinomas. P, positive control for *ras*12 mutation. N, negative control for *ras*12 mutation, i.e. non-carcinogen treated rat mammary gland DNA. The presence of the shorter band is diagnostic of the *ras*12 mutation.

tissue samples for this study. Female Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY) at 21 days of age and fed a modified AIN76A diet. At 50 days they were given an i.p. injection of MNU (Ash Stevens Inc., Detroit, MI) by the method reported by Thompson and Adlakha (6). The dose level was 37.5 and 25 mg MNU per kg body weight (BW) for experiments 1 and 2 respectively. The rats were palpated for mammary tumors twice per week. When a tumor was first palpated, the date and the tumor location were recorded. The experiments were terminated at 22 and 25 weeks post-carcinogen for experiments 1 and 2 respectively. At necropsy, tumors and suspicious lesions were excised, fixed in 10% neutral buffered formalin (12 h) and later embedded in paraffin and sectioned for histological evaluation. The pathological criteria were as described by Young and Hallowes (15). Only tumors that were classified as carcinomas were used for genotyping the *ras*12 status.

The paraffin-embedded tumor blocks were serially cut into 5-μm sections and were mounted on thin transparent plastic slides coated with polylysine (Sigma Chemical Company, St Louis, MO) and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked into distinct lobules for tissue retrieval (see examples in Figure 1A). A small piece (~2×2 mm) was carefully cut with flame-sterilized scissors from each marked area. Each piece was incubated with 10 μg proteinase K in 50 μl of 100 mM Tris–HCl, 2 mM EDTA at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 2–5 μl of the extract was used as the source of DNA for 40 cycles of PCR amplification.

The mutational status of *ras*12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5′AGTGTGATTCTCATTGGCAG-3′) was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene (17). The G→A mutation and two introduced mismatches in the downstream primer (5′-AGGGCACTCTTTCgaACGCC-3′, mismatches denoted by low case letters) generated an XmnI site in the PCR product (116 bp). Upon digestion of the product with XmnI (New England Biolabs, Beverly, MA), a fragment of 98 bp would be generated that was diagnostic for the mutation. A tracer amount of α-^{32}P-dCTP was used to label the PCR products. The digested products were separated by electrophoresis on a 6% polyacrylamide gel and detected by autoradiography as shown in Figure 1C.

Statistical methods used in the analyses of these experiments included descriptive statistics and χ^2-tests including Mantel–Haenszel tests for homogeneity of the association stratified by number of carcinomas per animal.

To address the first issue, 44 randomly selected carcinomas were analyzed. Of these carcinomas, 25 were sampled with two sites each, five with three sites each, and 14 with four sites each. Each site was genotyped for *ras*12 status (see examples in Figure 1C, rat234T6a-d, rat235T5a,b and rat22T1a-c). A total of 43 of 44 mammary carcinomas analyzed showed concordant *ras*12 (i.e. either all sites were mutant or all sites were wild type) among the multiple sites sampled (Table I). The exception was rat235 T5 in which the two sites were discordant for *ras*12. In fact, this observation initially prompted us to examine the issue of the origin of morphologically discernible lobules, which were often observed within carcinomas, especially in large ones.

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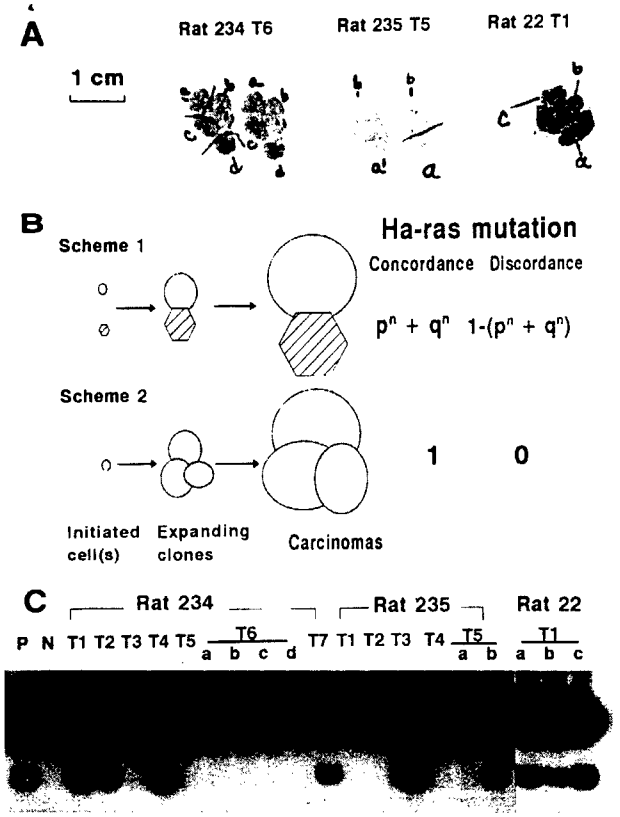

Fig. 1. (**A**) Examples of the gross appearance of tumor sections (H&E) on thin plastic slides. Tumors, especially large ones, were often observed to be made up of morphologically discernible lobules. The horizontal bar represents 1 cm in length. Sample code key: Rat234T6a, Rat #234, tumor #6, sampled site a. Lower case letters indicate sites from which tissue was sampled for *ras*12 genotyping. (**B**) Schematic illustration of multiple independent origins (polyclonality, Scheme 1) and a common origin of cancerous cells within a carcinoma (monoclonality, Scheme 2). (**C**) PCR-RFLP analysis of *ras*12 genotype in carcinomas. P, positive control for *ras*12 mutation. N, negative control for *ras*12 mutation, i.e. non-carcinogen treated rat mammary gland DNA. The presence of the shorter band is diagnostic of the *ras*12 mutation.

tissue samples for this study. Female Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY) at 21 days of age and fed a modified AIN76A diet. At 50 days they were given an i.p. injection of MNU (Ash Stevens Inc., Detroit, MI) by the method reported by Thompson and Adlakha (6). The dose level was 37.5 and 25 mg MNU per kg body weight (BW) for experiments 1 and 2 respectively. The rats were palpated for mammary tumors twice per week. When a tumor was first palpated, the date and the tumor location were recorded. The experiments were terminated at 22 and 25 weeks post-carcinogen for experiments 1 and 2 respectively. At necropsy, tumors and suspicious lesions were excised, fixed in 10% neutral buffered formalin (12 h) and later embedded in paraffin and sectioned for histological evaluation. The pathological criteria were as described by Young and Hallowes (15). Only tumors that were classified as carcinomas were used for genotyping the *ras*12 status.

The paraffin-embedded tumor blocks were serially cut into 5-μm sections and were mounted on thin transparent plastic slides coated with polylysine (Sigma Chemical Company, St Louis, MO) and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked into distinct lobules for tissue retrieval (see examples in Figure 1A). A small piece (~2×2 mm) was carefully cut with flame-sterilized scissors from each marked area. Each piece was incubated with 10 μg proteinase K in 50 μl of 100 mM Tris–HCl, 2 mM EDTA at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 2–5 μl of the extract was used as the source of DNA for 40 cycles of PCR amplification.

The mutational status of *ras*12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5′AGTGTGATTCTCATTGGCAG-3′) was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene (17). The G→A mutation and two introduced mismatches in the downstream primer (5′-AGGGCACTCTTTCgaACGCC-3′, mismatches denoted by low case letters) generated an XmnI site in the PCR product (116 bp). Upon digestion of the product with XmnI (New England Biolabs, Beverly, MA), a fragment of 98 bp would be generated that was diagnostic for the mutation. A tracer amount of α-^{32}P-dCTP was used to label the PCR products. The digested products were separated by electrophoresis on a 6% polyacrylamide gel and detected by autoradiography as shown in Figure 1C.

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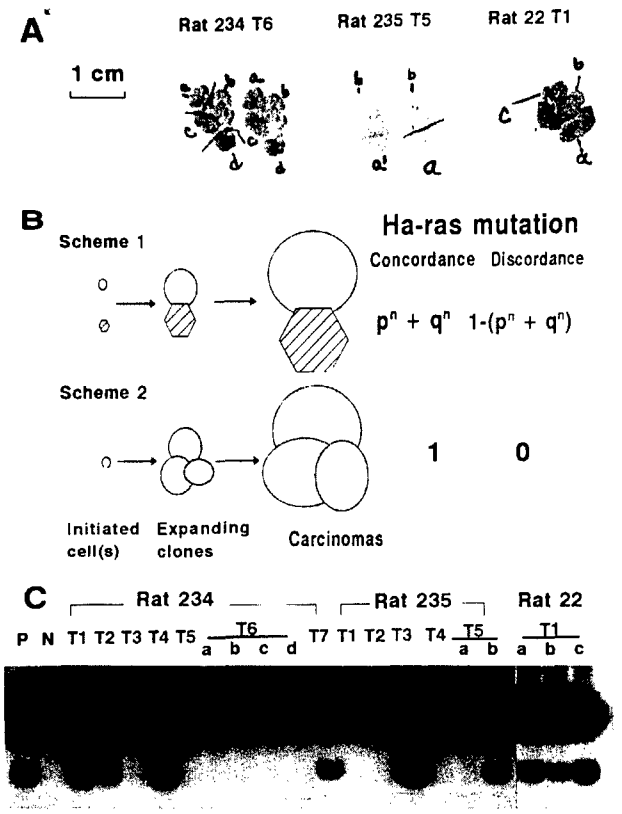

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Table I. Ha-*ras* codon 12 genotyping of multiple lobules of randomly selected mammary carcinomas from experiment 1

Number of sites analyzed per carcinoma (*n*)	Predicted *ras* concordance rate among sampled sites		Observed number of carcinomas with		Observed *ras*12 concordance rate among sampled sites
	Assuming polyclonality[a]	Assuming monoclonality[b]	concordant *ras* among sampled sites	discordant *ras* among sampled sites	
2	0.505	1	24	1[c]	0.96
3	0.258	1	5	0	1
4	0.132	1	14	0	1
Total		1	43		0.977

[a]Predicted *ras*12 concordance rate based on multiple, independent origins for cells in different lobules within a carcinoma (see Figure 1B, Scheme 1). The probability by chance for say 3 sites to show the same mutant *ras*12 genotype is $p \times p \times p$ and to show wild-type *ras*12 genotype is $q \times q \times q$, where p = probability for mutant *ras*12 and $q = 1 - p$ = probability for wild-type *ras*12 at a given site. Thus the overall concordance probability = $p^3 + q^3$. For n sites sampled, the predicted concordance is calculated by formula $p^n + q^n$. p was estimated by the overall frequency of mutant *ras*12 carcinomas and in this experiment, $p = 0.45$.

[b]Predicted based on monoclonal origin. The discordant *ras*12 genotypes among different sites is 0 because all sites will be either wild type or mutant at codon 12. The concordant rate is independent of the number of sites (n) sampled.

[c]This section (Rat 235 Tumor 5) displayed two distinctly H&E-stained regions. The discordant *ras*12 genotypes of the two portions sampled indicated that this section represented two independently initiated carcinomas growing together side-by-side.

treatments and lead to less than perfect templates for PCR. Fourth, the Taq polymerase used for PCR has a low but detectable level of amplification error per base incorporated (~0.02% with 20 cycles), which involves predominantly A→G transitions (21). Because the detection of the diagnostic mutant signal relies on the XmnI enzyme to recognize a six-base restriction sequence (...GAA*nnnn*TTC...), any amplification error in that sequence as a result of these latter two factors would lead to resistance to enzyme digestion of the PCR products, further reducing the mutant signal intensity. It was therefore reasoned that mutant *ras*12 signal ranging from 5% to 50% would be consistent with this mutation being an early marker in MNU induced mammary carcinogenesis. The diagnostic band intensity observed in both experiments was within this range.

With these factors taken into consideration, the high degree of *ras*12 concordance among multiple sites within a carcinoma (97.7%) strongly support Scheme 2 (Figure 1B), i.e. morphological heterogeneity, often manifesting as distinct lobes within a carcinoma, is likely the result of diversification of progeny cells of the original initiated cell during clonal expansion and subsequent progression as a carcinoma develops. A practical implication of this information is that tissue sampling for genotyping purposes, at least as far as the *ras*12 mutation is concerned, can be achieved by a single sample per tumor with good accuracy.

Do multiple carcinomas within the same animal share the same pathogenetic characteristics? If the answer to this question is yes, it should follow that multiple carcinomas within an animal will display concordant *ras*12 genotype because all carcinomas are either all mutant or all wild type. As shown in Figure 1C (rat234, T1-T7 and rat235, T1-T5) this was not the case. Table II tabulates the observed *ras*12 discordance rate as a function of the number of carcinomas borne by a rat. The data are consistent with the probabilities predicted based on independent origins among multiple carcinomas within the same animal (as illustrated in Figure 1B, Scheme 1). The result was observed in two independent experiments in which different amounts of carcinogen were used to induce mammary carcinogenesis. The independent nature of individual carcinomas within an animal supports the use of carcinoma multiplicity as a parameter for assessing the effects of preventive agents as well as risk factors. It should be noted, however, that the independent nature of initiation inferred here is true only at the molecular marker level. Our data do not rule out physiological (i.e. epigenetic) interdependence among carcinomas within the same animal. Such an epigenetic interaction among carcinomas or initiated cells can potentially result from changes in the endocrine factors and metabolic milieu brought about by a preexisting carcinoma and could influence the emergence of additional carcinomas in the same animal and/or the latency of their appearance. In an early study with this model, the kinetics of appearance of additional carcinomas was observed to slow down significantly after the appearance of the first carcinoma (2). The implication of a secreted inhibitory factor from a primary tumor in suppressing the emergence of secondary tumors (18) might account for this observation.

To address the issue of topographic location of mutant *ras*12 carcinomas with respect to the mammary gland chains, Table III summarizes the prevalence of wild-type and mutant carcinomas arising in the cervical-thoracic (C-T) and the abdominal-inguinal (A-I) glands. A significant regional difference in total carcinoma occurrence was observed between the C-T and the A-I glands in that there were approximately twice as many carcinomas in the former as in the latter glands, which is consistent with previous reports (1,5,6,19). But surprisingly, more than three times as many mutant *ras*12 carcinomas were located in the C-T glands as in the A-I glands, whereas the wild-type *ras*12 carcinomas were almost equally distributed between the two regions (Table III). The disproportional distribution pattern held true upon secondary analyses stratifying by the number of carcinomas per animal and by experiment. In fact, the previously observed 2:1 C-T to A-I ratio of carcinoma occurrence (1,5,6,19) could be almost entirely attributed to this preferential localization of mutant *ras*12 carcinomas in the C-T mammary gland chains. Whether this difference is related to the asynchronous post-natal development of the C-T versus and A-I glands (19) remains to be determined. Nonetheless, the practical implication of the observed regional differences should not be overlooked. Until the cause and the biological significance of the regional differences observed in this study are clearly understood, it is advisable to follow a consistent tissue collection protocol with respect to carcinoma location in the mammary gland chains so that this source of bias is minimized when carcinoma tissues

Table II. Ha-*ras* genotype profile of multiple mammary carcinomas within the same animals

Number of carcinomas per rat (*N*)	Predicted *ras* discordance rate among multiple carcinomas assuming independent origin[a]	Number of rats with concordant *ras* genotypes among carcinomas	Number of rats with discordant *ras* genotypes among carcinomas	Total number of rats in category	Observed *ras* discordance rate among multiple carcinomas
Experiment 1 (37.5 mg MNU per kg)					
2	0.495	11	10	21	0.476
3	0.742	3	8	11	0.727
4	0.868	0	8	8	1
5	0.931	0	11	11	1
6	0.964	1	9	10	0.9
7 or greater	>0.981	0	17	17	1
Experiment 2 (25 mg MNU per kg)					
2	0.442	22	22	44	0.5
3	0.663	8	12	20	0.6
4	0.787	4	11	15	0.73
5	0.861	2	4	6	0.67
6 or greater	>0.908	0	3	3	1

[a]Predicted discordance rate among multiple carcinomas borne by the same animal assuming pathogenetic independence. Calculated by formula $1 - (p^N + q^N)$, where p was estimated by the overall *ras*12 mutation frequency in carcinomas. $p = 0.45$, $q = 1 - p = 0.55$ in experiment 1 and $p = 0.67$, $q = 0.33$ in experiment 2 respectively. N = number of carcinomas per rat.

Table III. Distribution of mutant and wild-type *ras*12 mammary carcinomas by anatomical regions

Location of glands	Number of carcinomas with		Total	% Ha-*ras* mutation	χ^2, *P*-value[a]
	mutant *ras*12	wild type *ras*12			
Experiment 1 (37.5 mg MNU per kg)					
Cervical-thoracic chains	132	115	247	53	
Abdominal-inguinal chains	41	91	132	31	
Total	173	206	379	45	17.4 ($P < 0.005$)
Experiment 2 (25 mg MNU per kg)					
Cervical-thoracic chains	171	61	232	74	
Abdominal-iguinal chains	54	51	105	51	
Total	225	112	337	67	16.1 ($P < 0.005$)

[a]2×2 contingency table analysis, degree of freedom = 1. The strong association between the anatomical region and occurrence of mutant *ras*12 carcinomas observed in both experiments were further examined by stratifying over the total number of carcinomas per animal and by experiment. The overall Cochran–Mantel–Haenszel $\chi^2 = 33$, $P < 0.001$. The disproportional pattern of mutant *ras*12 carcinoma occurrence was observed for each of the 10 strata in experiment 1, and 7 out of 8 strata in experiment 2. The probability for such observed disproportional distribution occurring by chance is $P < 0.01$. This secondary analyses did not support the existence of bias of the distribution pattern due to carcinoma multiplicity per animal.

are collected for biochemical and cytological assessment. The sampling issue is especially significant when 'gene-specific' prevention of subpopulations of pathogenetically identifiable neoplasia is concerned. For such applications of molecular markers, it is imperative that identifiable cancerous lesions from every gland be genotyped.

The overall *ras*12 mutation frequency in mammary carcinomas was 45% at a MNU dose of 37.5 mg/kg and 67% at 25 mg/kg (Table III). These results confirmed an earlier report that the percentage of mutant *ras*12 carcinomas was inversely related to the dose of MNU (10). That study also reported the disproportional increase in wild-type *ras*12 carcinomas in rats with experimental hyperprolactinemia (10). We have reported that the incidence of wild-type *ras*12 carcinomas could be increased preferentially by dietary risk factors over those with the mutation (17,20). These studies highlight the potential importance of risk assessment based on a knowledge of the pathogenetic characteristics of the disease.

In summary, experimental data presented in this study were consistent with the clonal evolution of multiple, independently-initiated cells giving rise to distinct mammary carcinomas in the same animal, and pointed to a significant topographic difference in the occurrence of mutant *ras*12 carcinomas between the C-T and the A-I mammary glands. These observations support the validity of statistical tests based on the assumption of independent emergence of lesions for the evaluation of the carcinogenesis data in this model and they further stress the need of representative sampling with gland location to be taken into consideration.

Acknowledgements

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Paper 13

Telomerase Activity in the Normal and Neoplastic Rat Mammary Gland[1]

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ABSTRACT

The 1-methyl-1-nitrosourea-induced rat mammary tumor model system is well studied, reproducible, and widely used. We have investigated whether these tumors possess higher telomerase activity than normal mammary tissue. Using the telomeric repeat amplification protocol assay, we found significantly higher telomerase activity in 36 mammary carcinomas than in 72 mammary glands of virgin rats. The level of telomerase activity in virgin rats was unaffected by strain, age, stage of the estrous cycle, or ovariectomy. However, mammary glands obtained from pregnant rats exhibited telomerase activity comparable to that found in the tumors, possibly reflecting the high epithelial content of these tissues. Indeed, isolated epithelial cells from virgin and pregnant mammary glands and from carcinomas had similar telomerase activities. Thus, telomerase activity is constitutive in the rat mammary epithelium and is not a unique characteristic of malignant transformation in this tissue. These results underscore the importance of attributing biochemical properties to specific cell types in a tissue, a situation not paralleled in the interpretation of data from *in vitro* models.

INTRODUCTION

The ribonucleoprotein telomerase is responsible for the synthesis and maintenance of the specialized structures at the ends of chromosomes called telomeres, which display many important structural and functional roles during interphase, mitosis, and meiosis (1, 2). Telomeres consist of tandemly repeated DNA sequences (TTAGGG) ranging from 5–20 kb in length in humans, 20–100 kb in length in the rat, and 100–150 kb in mice (3). They protect genomic DNA from degradation and deleterious recombination events (1, 2). Although telomerase is not usually detectable in normal somatic differentiated tissues, it appears to be reactivated in a large variety of human tumor types, resulting in the reinitiation of synthesis of telomere repeat units, stabilizing telomere length (4, 5). Recent studies have shown that, in contrast to humans, almost all somatic tissues of mice and rats (6–13) have detectable telomerase activity. The presence of telomerase activity in normal rat tissues was attributed to the presence of somatic stem cells in the tissues with regeneration potential, an idea similar to that reported in human epithelium (14), skin (15), and hematopoetic cells (16). The level of telomerase activity was, however, substantially higher in the rodent tumors compared to the corresponding normal tissues (6–12). In this study, we have evaluated telomerase activity as a tumor biomarker in rat mammary gland and MNU[4]-induced mammary carcinomas (17, 18), a model system widely used in experimental carcinogenesis research (19, 20). Based on the work cited above, we hypothesized that higher telomerase activity would be observed in mammary carcinomas than the normal mammary gland, and that the degree of increase would be a consistent phenotype that would distinguish carcinomatous mammary epithelium from nontransformed mammary epithelium. As expected, we found high telomerase activity in mammary carcinomas. However, further investigation revealed that enzyme activity in mammary carcinomas may be a reflection of the high epithelial cell content of the tissue and that telomerase activation is not an obligatory event of malignant transformation in this system.

MATERIALS AND METHODS

Tissues. For determining telomerase activity in normal mammary glands, six female Wistar-Furth rats were used. This group consisted of three 13–15-week-old parous rats, and 3 28-day-old virgin rats. Female Sprague Dawley rats were used for studying the influence of age, parity, stage of the estrous cycle, hormonal modulation, pregnancy, and lactation on telomerase activity in breast tissue. The impact of age on telomerase activity was tested in 30-, 60- and 90-day-old virgin rats. To test telomerase activity caused by hormonal changes in the breast, mammary glands from six 10-week-old parous rats, four 35-day-old virgin rats at estrus and diestrus (2-day timed cycle), eight 10-week-old rats at early, mid, and late pregnancy, and six estrogen-reconstituted ovariectomized rats were examined. Ovariectomy was performed on six 50-day-old female rats. Four days after surgery, three rats were treated with one dose of 17β-estradiol (5 μg/0.1 ml corn oil/rat) s.c., whereas the remaining three received the vehicle. Rats were sacrificed 76 h after initiation of hormone treatment. All 12 mammary glands from each rat were snap frozen in liquid nitrogen and stored at −80°C. For comparison of telomerase in mammary glands of various strains, mammary glands were collected from 30-day-old female virgin rats of strains Sprague Dawley, Wistar Furth, Fisher 344, Copenhagen, Lewis, and Buf/N. Cell suspensions were prepared from the six mammary glands on the right side by digesting the minced tissue with collagenase (800 units) and hyaluronidase (200 units) and enriching for epithelial cells as described (21). The digest was filtered; the organoids were collected off the filter and stored frozen at −80°C. The remaining six mammary glands were snap frozen and stored at −80°C. Identical digests were obtained from three pregnant (20-day) rat mammary glands.

Mammary Tumors. Female Sprague Dawley or Wistar-Furth rats (45 days old) received a single i.p. administration of MNU (50 mg/kg body weight; Ash-Stevens). Tumors arose in MNU-treated rats 2–4 months later (17, 18). Tissues were excised, trimmed, and frozen at −80°C. Mammary glands containing small tumors (<10 mm^3) from 13 rats were embedded in OCT and cryosectioned; islands of tumor cells and normal ductal epithelium from tumor-free glands were microdissected from three to four consecutive sections under ×10, using an 18-gauge hypodermic needle, and placed in lysis buffer (see below).

TRAP Assay on Tissue and Cell Lysates. The PCR-based TRAP assay for telomerase activity was used as described previously (22, 23). Ten-μm frozen sections were obtained of tissues embedded in OCT compound. One section from each tissue was stained with H&E for histological confirmation. Telomerase activity was determined in duplicate on lysates of 5–10 adjacent sections by the TRAP assay (22), and a negative control was provided for each extract by heat inactivation at 95°C for 10 min. To enable quantitation of telomerase activity levels, telomerase reactions were repeated using the TRAPeze assay kit as described (Ref. 23; Oncor, Gaithersburg, MD); each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard. The level of telomerase in each extract was determined by measuring the combined intensities of the radioactive signal for each sample and comparing it with that obtained by using a fixed amount of protein from the quantitation standard (23). In this study, instead of the quantitation controls

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[4] The abbreviations used are: MNU, 1-methyl-1-nitrosourea; ITAS, internal TRAP assay standard; TRAP, telomeric repeat amplification protocol.

provided in the kit, we used one to three reactions of 0.06 μg of protein from lysates of a mouse mammary tumor cell line, MOD, per set of reactions. Tissue lysates were tested using between 0.06 and 2.0 μg of protein per reaction, depending upon the strength of the telomerase reaction following a 12–24-h exposure of the autoradiograph at −70°C. Each set of reactions included tubes without any extract, and extracts were heated at 95°C for 10 min or treated with RNase A (200 μg/ml). The average absorbance of the first eight TRAP bands above the primer band was calculated as a ratio to the ITAS. Quantitation was performed using the IP Lab Gel software. We used an arbitrary unit for quantitation of telomerase activity in our samples, which was derived for each sample as follows:

$$\text{Relative telomerase activity} = \frac{TE/I/TE^*/I}{ME/I/ME^*/I}$$

where *TE*, *ME*, and *I* is the intensity of the signal in the tissue extract (2.0 μg of protein), in the MOD cell extract (0.6 μg protein), and in the ITAS standard, respectively. The * denotes extracts where telomerase has been heat inactivated prior to initiation of the reaction. None of the extracts with negative and low level telomerase activity inhibited the telomerase activity of MOD, excluding the presence of an inhibitor to telomerase in these extracts.

Statistical Analysis. All *P*s were derived using Wilcoxon's Rank Sum test for nonparametric data; $P < 0.05$ was considered statistically significant. The statistical analysis was performed using the JMP statistical software package (SAS Institute, Inc.) on a Macintosh microcomputer.

RESULTS

Telomerase Activity Is Detectable in Mammary Glands from Different Strains of Rat. Several rat strains are commonly used in the investigation of chemically induced mammary carcinogenesis. Thus, we first sought to determine if constitutive levels of telomerase could be detected in the mammary glands of the most commonly used rat strains. We compared the telomerase activity in two whole mammary gland lysates from two rats each of six different strains: Sprague Dawley, Wistar/Furth, Lewis, Buf/N, Fisher 344, and Copenhagen. A low level of telomerase activity was observed in the lysates prepared from pooled sections (from six mammary glands each) of the six rat strains (data not shown). In view of this observation, we proceeded to evaluate constitutive levels of telomerase more extensively in both parous and virgin Wistar-Furth rats because this a commonly used inbred rat strain in experimental mammary carcinogenesis. As shown in Fig. 1, detectable activity was readily observed in several among the 72 mammary glands derived from six rats, irrespective of their anatomical location. However, telomerase activity in the parous rats was significantly lower than in the 28-day-old virgin rats ($P < 0.01$). Although activity was variable among glands, no consistent pattern to these differences was observed. The majority of the mammary glands from both sets of rats, however, expressed telomerase activity at low or undetectable levels (Fig. 1).

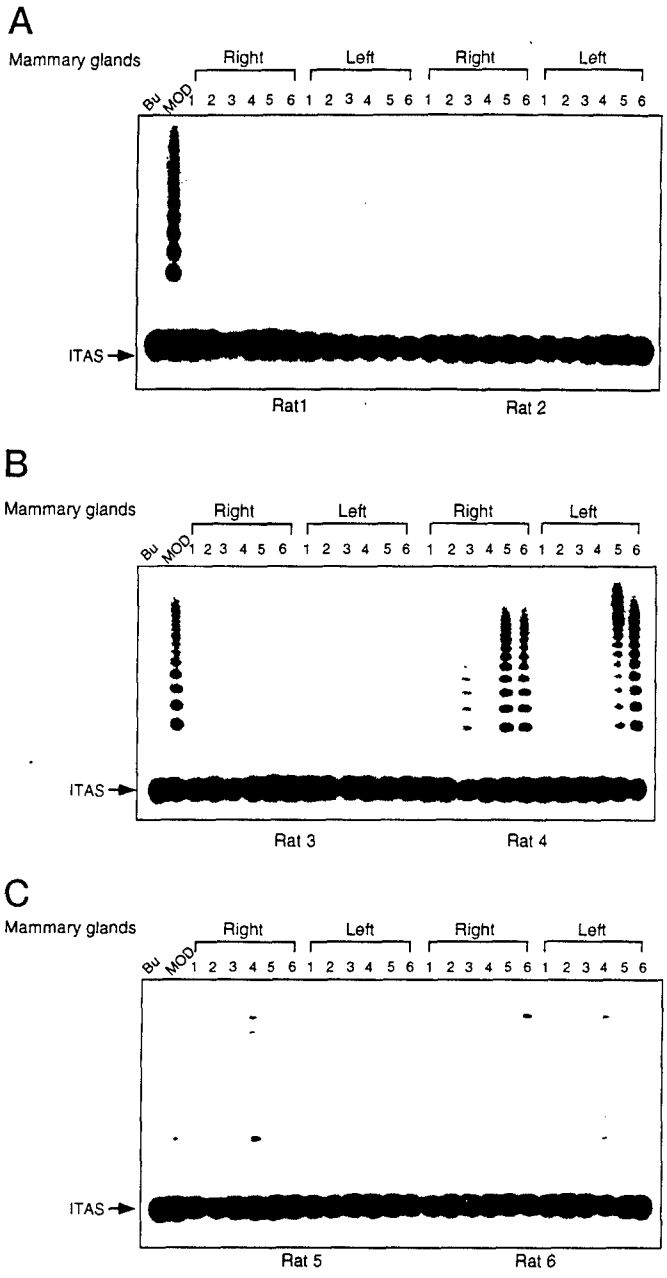

Fig. 1. TRAP assay of telomerase activity in normal rat mammary gland. Whole-tissue extracts (2.0 μg of protein) prepared from each of 12 mammary glands from rats 1–6 were tested using a commercial assay kit (TRAPeze; Oncor). *Rat 1–3*, Rat 13–15-week-old parous rats; *Rat 4–6*, Rat 28-day-old virgin rats. *Bu*, lysis buffer, negative control; *MOD*, 0.06 μg of protein from mouse mammary cell line; *ITAS*, 36-bp ITAS.

Telomerase Activity Is High But Variable in MNU-induced Mammary Carcinomas in Rats. Levels of telomerase activity were evaluated in 36 mammary carcinomas induced by MNU. All carcinomas were positive for telomerase activity, although the level of activity varied greatly among individual carcinomas (Fig. 2 and data not shown). Quantitative analysis of the telomerase activity present in the tumors was performed to determine the telomerase activity in the tumor extract relative to the normal mammary gland. Telomerase levels in the mammary carcinomas were significantly higher than in the normal mammary gland ($P < 0.0001$), suggesting the potential utility of telomerase as a tumor marker.

Is Elevated Telomerase Activity a Tumor-specific Phenotype? Based on the observation that telomerase activity is low in normal mammary glands but high in mammary carcinomas, we asked whether the increased levels were associated with tumorigenesis or whether they reflected changes in growth and architecture of the mammary gland in response to hormonal stimuli. To test the effects of hormonal modulations of mammary growth, tissue lysates from two mammary glands each of rats: in diestrus and estrus; ovariectomized; ovariectomized followed by exogenous estrogen; and at early, mid, mid/late stages of pregnancy were examined for telomerase activity. Mammary glands of rats in estrus showed a slightly higher telomerase activity compared to rats in diestrus (Fig. 3*A*). A similar increase was observed in the mammary glands of ovariectomized rats 48 h after estrogen administration (Fig. 3*A*). However, telomerase activity in mammary glands of rats at early, mid, or late stages of pregnancy (Fig. 3*B*) was significantly higher than in virgin rats ($P < 0.0001$). In fact, the range and distribution of activities in the pregnant gland was similar to that observed for mammary tumors (Fig. 2*B*).

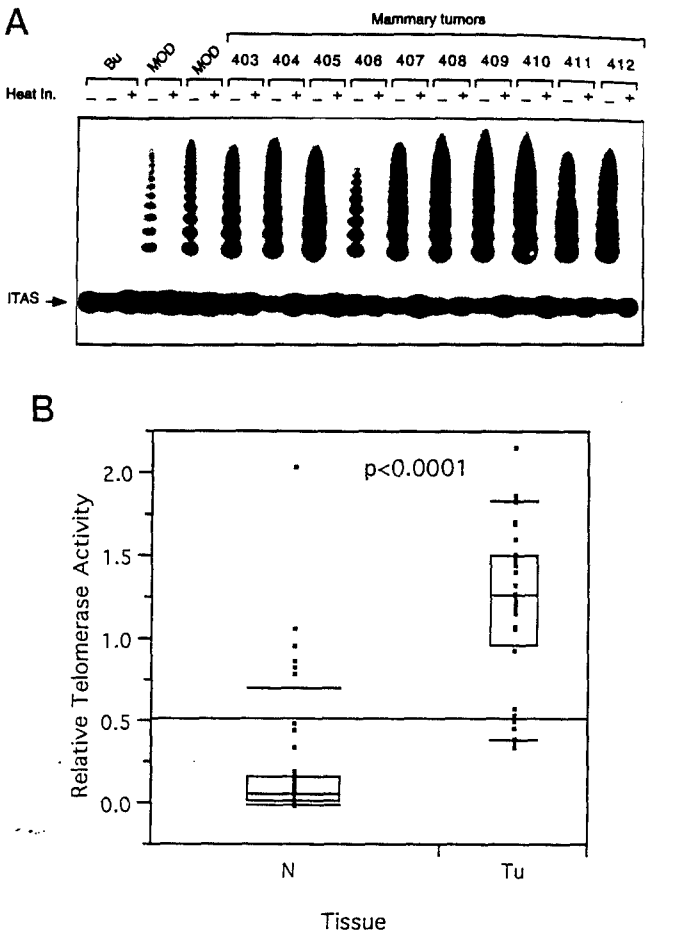

Fig. 2. *A*, telomerase activity in MNU-induced rat mammary carcinomas. *Bu*, lysis buffer; *MOD*, mouse mammary tumor cells (0.06 μg of protein). Lysates (2.0 μg) prepared from pooled cryosections of mammary tumors were subjected to TRAP (TRAPeze) assay. Samples were subjected to the TRAP assay with (+) or without (−) heat inactivation of the tissue extract. *B*, nonparametric Wilcoxon Tests (Rank Sums) were performed comparing *Relative Telomerase Activity* present in 76 normal mammary glands (*N*) to that present in 36 MNU-induced mammary tumors (*T*) using a JMP Statistics program (SAS Institute, Inc.). Quantile box plots for each group are shown, summarizing the distribution of data points for both groups; the *top* and *bottom* of the *box* represent the 75th and 25th quantile levels, respectively, whereas the upper line and lower lines represent the 90th and 10th quantile levels, respectively.

The major structural difference between virgin and pregnant mammary gland is the ratio of stroma to the epithelium. Whereas epithelial ducts are infrequently distributed in an abundance of stroma in the virgin mammary gland, the reverse is true in the pregnant mammary gland (24). We, therefore, asked whether the differences in telomerase activity could be attributed to the cellular composition of the tissue. We determined telomerase activity in lysates from epithelial organoids obtained by collagenase digestion of six mammary glands each from six pubertal and three pregnant rats. Lysates of organoids from virgin and pregnant mammary glands (Fig. 4*A*) showed comparably high telomerase activity.

The analysis of telomerase activity in the mammary organoids established that the epithelial cell compartment in the mammary gland was, in all probability, the source of telomerase activity. Therefore, variations in the ratio of epithelium to stroma in tissue samples can lead to large variations in measured telomerase activity in tissue samples. This result, however, did not explain the wide range of telomerase activity observed in the tumors that are predominantly composed of epithelial cells (Fig. 2*B*). To determine whether this variation in telomerase activity is a reflection of the heterogeneity of epithelial cell populations in the mammary gland, we tested 24 microdissected carcinoma loci and 24 histologically normal ductolobular units from an uninvolved gland from the same rat. Telomerase activity in the carcinomas and normal ductal samples varied widely from undetectable activity to very high activity (Fig. 4*B*). These results indicate that different ductolobular units in the breast (as well as carcinomas) express different levels of telomerase activity, a fact that was not readily apparent when pooled organoids were tested (Fig. 4*A*).

DISCUSSION

In this study, we provide evidence that telomerase activity is constitutive in rat mammary epithelial cells. Telomerase activity of whole tissue mammary gland homogenates from normal virgin rats was very low compared to activity in MNU-induced mammary carcinomas. However, we showed that this difference could be attributed to the vast difference in epithelial:stromal ratio between the two, because epithelial cells isolated from the mammary gland expressed the same level of telomerase activity as the mammary carcinoma.

The objective of this study was to test the hypothesis that elevated telomerase activity would be a distinguishing characteristic of mammary cancer. To provide a basis for hypothesis testing, the constitutive levels of telomerase in virgin mammary glands from animals of various ages and different rats strains were assessed. In all cases, telomerase activity was detected. This finding is consistent with reports of detectable levels of telomerase in other tissues of the rat (10–13) and with data from mice (6–9, 12). We then proceeded to assess telomerase activity in a randomly selected subset of MNU-induced rat mammary carcinomas (17, 18). A comparison of telomerase activity in whole-tissue lysates of virgin mammary glands (Fig. 1) to telomerase activity in MNU-induced mammary tumors (Fig. 2) provided strong support for the hypothesis that telomerase activity is

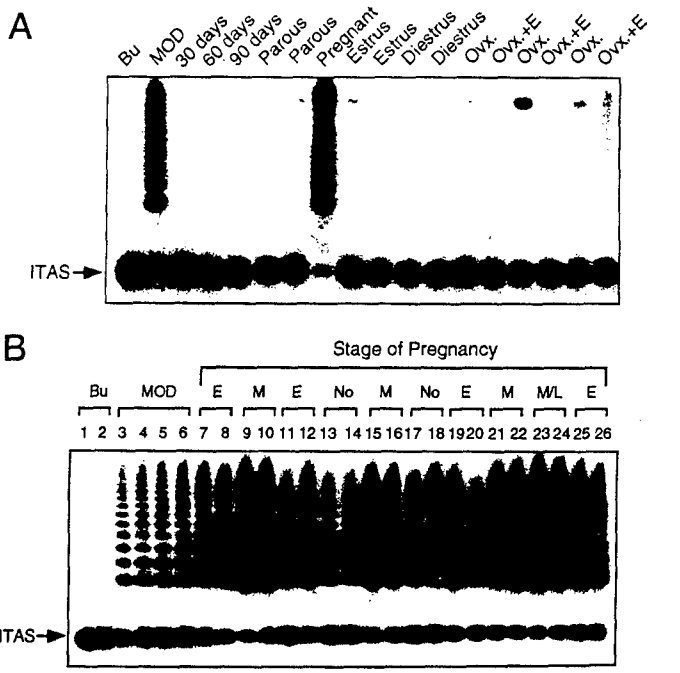

Fig. 3. *A*, influence of age, parity, estrus cycle, pregnancy, and ovariectomy (*Ovx.*) on telomerase activity. *Bu*, lysis buffer; MOD, mouse mammary tumor cells (0.06 μg of protein); E, estrogen. Protein (2.0 μg) of the indicated tissue lysate was used for the TRAP (TRAPeze; Oncor) assay. *B*, influence of pregnancy on telomerase activity. *Bu*, lysis buffer; *MOD*, mouse mammary tumor cells (0.06 μg of protein). Protein (2.0 μg) of the indicated tissue lysate was used for the TRAP (TRAPeze, Oncor) assay. *No*, normal virgin mammary gland from 30-day-old rat; *E*, early pregnancy, 5–7 days postcoitum; *M*, mid-pregnancy, 10–12 days postcoitum; *M/L*, mid/late, 13–18 days postcoitum; *L*, late pregnancy, 19–21 days postcoitum. ITAS, 36-bp ITAS.

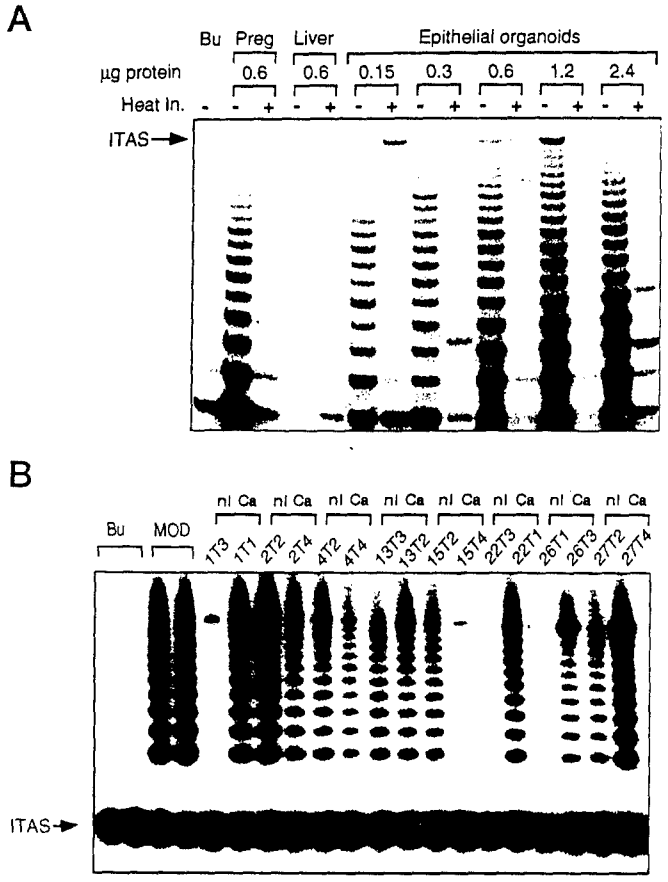

Fig. 4. *A*, telomerase activity in epithelial organoids isolated from rat mammary glands. *Bu*, lysis buffer; *Preg*, organoids isolated from 20-day pregnant rat mammary glands; *Liver*, liver from 30-day-old female rat. Epithelial organoids were obtained by digestion of mammary glands from 30-day-old Sprague Dawley rats. Samples were subjected to the TRAP assay (22, 23) with (+) or without (−) heat inactivation (*Heat In.*) of the tissue extract. *ITAS*, 150-bp ITAS. *B*, telomerase activity in microdissected normal lobules and tumor foci in the mammary glands of MNU-treated rats. *Bu*, lysis buffer; *MOD*, mouse mammary tumor cells (0.06 µg of protein). Extracts (0.12 µg of protein) of microdissected cells from normal ducts (*nl*) or carcinoma (*Ca*) were subjected to TRAP (TRAPeze; Oncor) assays. *ITAS*, 36-bp ITAS.

higher in mammary carcinomas than in virgin mammary gland. This observation set the stage for further rigorous testing of telomerase as an intermediate tumor marker.

One of the fundamental questions in telomerase biology is whether telomerase activation occurs during rapid growth in response to mitogenic signals issued as a result of normal physiological processes or whether it occurs in response to signals specific to neoplastic transformation. Telomerase activity was not significantly affected by stage of the estrous cycle or the administration of exogenous estrogen (Fig. 3*A*), consistent with the notion that telomerase activation is specific to neoplastic transformation. However, the high levels of telomerase observed in the pregnant mammary gland (Fig. 3*B*) strongly argued against this hypothesis. In fact, the telomerase activity levels in pregnant mammary glands were as high as those seen in many mammary carcinomas. We reasoned that elevated telomerase activity might be a reflection of a change in the epithelial cell compartment of the tissues. Whereas the virgin mammary gland is mainly composed of adipose tissue and stroma interspersed with a few epithelial ductal lobular units, in the pregnant mammary gland there is an expansion in the number of ductal lobular units, resulting in a drastic increase (up to 70%) in the proportion of epithelial cells relative to stroma and fat cells. The mammary carcinomas, even more so than the mammary glands of pregnant rats, are predominantly epithelial in content but can contain varying numbers of interlacing stromal cells (24). Mammary organoids isolated by enzymatic digestion of pregnant mammary glands showed telomerase activity comparable to that present in the virgin mammary gland from all six rat strains: Sprague Dawley, Wistar Furth, Fisher 344, Copenhagen, Lewis, and Buf/N (Fig. 4*A* and data not shown). These findings argue strongly that telomerase activity is constitutive in mammary epithelial cells. Constitutive activity has also been observed in rat colon (10), liver (10, 11, 13), brain (12), vagina, mammary gland, and uterus[5] and in myometrium, as well as in leimyomas, tumors arising from the myometrium of the uterus.[6]

Although the analysis on organoids represents the activity present in a particular cell type compartment in the mammary gland, it is nevertheless a pooled population of epithelial elements. We, therefore, subjected the question to more rigorous testing by measuring telomerase activity in microdissected specimens of both tumor and normal epithelial islands. We found a great variability in telomerase levels among the samples (Fig. 4*B*). This was not a characteristic unique to tumors, because similar variations were observed in microdissected islands of epithelial cells from normal glands as well (Fig. 4*B*). Thus, we can conclude that the constitutive telomerase activity present in the rat mammary epithelium is highly variable. Although the most obvious explanation for this variability is differences in the proportion of stem cells in the population, our poor understanding of mammary cell biology prevents us from speculating any further.

Reactivation of telomerase appears to be essential for maintenance of telomere length in human tumor cells (1–5). By contrast, as noted above, telomerase activity is constitutively high in many normal rodent tissues. For this reason, it seems unlikely that reactivation of telomerase is necessary for malignant transformation in rodents. However, several groups have reported a quantitative difference between telomerase activity in rodent tumors of the skin, pancreas, mammary gland, and liver when compared to normal somatic tissues (7–9, 11, 12). It appears that these investigators have not taken into account the difference in the dense epithelial cellular composition of the tumor compared to the relatively infrequent presence of epithelial cells in the normal tissue. In view of our observations, a similar detailed study is warranted in these tumor model systems as well as in human breast cancer (25, 26).

In summary, telomerase activity was significantly elevated in mammary tumors and mammary gland from pregnant animals, effects related to the increased ratio of epithelial to stromal cells in these two tissues. Although telomerase is not a marker of transformation *per se*, it remains to be determined whether the variability in the telomerase activity of morphologically similar carcinomas will be an informative tumor endpoint biomarker of prognostic value. These results underscore the importance, in interpreting telomerase activity data derived from tissues, of taking into account differences in the epithelial: stromal ratio of cells in the biopsied tissue, a situation not paralleled in the interpretation of data from *in vitro* models.

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